Inhibition of the crosstalk between dendritic, natural killer and T cells by mesenchymal stromal/ stem cells

D i s s e r t a t i o n

zur Erlangung des akademischen Grades

d o c t o r r e r u m n a t u r a l i u m (Dr. rer. nat.)

im Fach Biologie

eingereicht an der

Lebenswissenschaftlichen Fakultät
der Humboldt-Universität zu Berlin

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Tag der mündlichen Prüfung: 28.09.2016

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SUMMARY

Mesenchymal stromal cells (MSC) directly and indirectly support endogenous tissue regeneration and are promising candidates for regenerative therapies targeting acute or chronic ischemic tissue injuries and immune-mediated pathogenic processes. Additionally, MSC seem to be low immunogenic, which allows application across major histocompatibility complex (MHC) barriers, even though contradictory results are described. But little is known about the mechanisms for their immune escape and immunomodulation. Hence, the main goal of the presented study was to understand if MSC interfere with the crosstalk between dendritic cells (DC), natural killer (NK) cells and T cells by influencing DC maturation, since this network is important for efficient priming of naïve T cells into type 1 helper T cells (Th1).

Firstly, it is shown that bone marrow-derived MSC (BM-MSC) had diverse effects on the maturation of different human DC subsets in vitro, depending on the type of DC and their time of interaction. BM-MSC inhibited differentiation but not maturation of monocyte-derived DC (moDC). They did not have a clear effect on maturation of plasmacytoid DC (pDC), whereas they induced a tolerogenic phenotype in activated CD1c+ myeloid DC (mDC). This was characterized by an impaired CCR7-dependent migration and an anti-inflammatory cytokine profile. As a consequence, BM-MSC-licensed mDC displayed a reduced ability to induce interferon (IFN) g production in NK cells due to their altered interleukin (IL)-12/IL-10 secretion. BM-MSC-licensed mDC were also less efficient in inducing lineage commitment of naïve T cells towards the Th1 compartment. Similar in vitro results were observed with placenta-derived mesenchymal-like adherent stromal cells (PLX-PAD).

Finally, samples from critical limb ischemia (CLI) patients treated with an off-the-shelf MSC-like cell product within a phase I/IIa clinical trial were analysed for alloimmunogenicity of PLX-PAD. None of the patients developed a significant memory T cell response specific to the unmatched allogeneic cell product. The low immunogenicity observed after in vivo application might be explainable by our in vitro observations.

It is difficult to detect MSC in tissues because they lack specific lineage markers, which is why multiparameter combinations are needed. In this work, it is shown that CD73+ CD90+ CD105+ CD45- CD34- CD14- CD19- MSC could be detected in human placenta cryosections using a novel multiplex-immunohistology technique (chipcytometry). This offers the possibility to investigate the crosstalk between injected MSC and attracted immune cells, such as DC and NK cells, in patient biopsies in the future.

ZUSAMMENFASSUNG

Mesenchymale Stromazellen (MSC) unterstützen die endogene Geweberegeneration auf direktem und indirektem Weg. Dadurch bieten sie eine vielversprechende Möglichkeit für regenerative Therapien zur Behandlung akuter oder chronischer ischämischer Gewebeschäden und immunvermittelter pathogener Prozesse. Obwohl zum Teil kontroverse Ergebnisse diskutiert wurden, scheinen MSC nur schwach immunogen zu sein. Dadurch ist die Anwendung eines allogenen Zellproduktes trotz abweichendem Haupthisto-kompatibilitätskomplex (MHC) möglich. Es ist jedoch wenig darüber bekannt, wie die geringe Immunogenität und die Immunmodulation zustande kommen. Das Hauptziel der vorliegenden Arbeit war es zu untersuchen, ob MSC in die Interaktion zwischen Dendritischen Zellen (DC), Natürlichen Killer (NK) Zellen und T Zellen eingreifen, indem sie die DC-Reifung beeinflussen. Dieses Netzwerk ist wichtig für eine effiziente Differenzierung naïver T Zellen zu Typ 1 T Helferzellen (Th1).

Zunächst wird gezeigt, dass aus dem Knochenmark isolierte MSC (BM-MSC) unterschiedliche Auswirkungen auf die *in vitro* Reifung verschiedener DC Subtypen hatten. Der Effekt war abhängig von der DC-Art und dem Zeitpunkt des Aufeinandertreffens. BM-MSC inhibierten die Differenzierung, aber nicht die Reifung humaner von Monozyten-abgeleiteter DC (moDC). Weiterhin hatten BM-MSC keinen eindeutigen Einfluss auf die Reifung plasmazytoider DC (pDC), wohingegen sie in aktivierten $CD1c^+$ myeloiden DC (mDC) einen tolerogenen Phänotyp induzierten. Dieser war insbesondere durch eine schlechtere CCR7-abhängige Migration und ein anti-inflammatorisches Zytokinprofil charakterisiert. Daraus resultierend, wiesen mDC, welche in Gegenwart von BM-MSC maturiert wurden, aufgrund ihrer veränderten Interleukin (IL)-12/IL-10 Sekretion eine geringere Fähigkeit auf, die IFNγ Produktion von NK Zellen zu stimulieren. Diese BM-MSC-geprägten mDC induzierten ebenfalls weniger effizient die Differenzierung naïver T Zellen zu Th1 Helferzellen. Vergleichbare *in vitro* Ergebnisse wurden mit *placenta-derived mesenchymal-like adherent stromal cells* (PLX-PAD) beobachtet.

Ferner konnte in dieser Arbeit keine Alloimmunogenität in Patienten mit kritischer Ischämie der Extremitäten (CLI), welche im Rahmen einer Phase I/IIa klinischen Studie

allogene PLX-PAD erhalten hatten, nachgewiesen werden. Keiner der Patienten entwickelte eine signifikante Gedächtnis T Zellantwort spezifisch für das allogene MSC-ähnliche Standardzellprodukt. Die geringe *in vivo* Immunogenität könnte durch unsere *in vitro* Beobachtungen erklärbar sein.

Da es keine spezifischen MSC-Marker gibt, ist es schwierig MSC im Gewebe nachzuweisen, wodurch Markerkombinationen notwendig sind. In dieser Arbeit konnte gezeigt werden, dass $CD73^+$ $CD90^+$ $CD105^+$ $CD45^-$ $CD34^-$ $CD14^-$ $CD19^-$ MSC mithilfe einer neuen Multiplex-Immunhistologie-Technik (Chipzytometrie) in humanen Plazenta-Kryostatschnitten detektiertierbar sind. Für die Zukunft bietet diese Methode die Möglichkeit in Biopsien zu untersuchen wie injizierte MSC mit den angelockten Immunzellen, zum Beispiel DC und NK Zellen, interagieren.

ABBREVIATIONS

ABI	ankle-brachial index
APC	antigen presenting cells
ATP	adenosine triphosphate
auto	autologous
BCR	B cell receptor
BDCA	blood dendritic cell antigens
bFGF	basic fibroblast growth factor
BM-MSC	bone marrow-derived mesenchymal stromal/stem cells
(BM-MSC)mDC	mDC matured in the presence of BM-MSC
(BM-MSC)pDC	pDC matured in the presence of BM-MSC
BSA	bovine serum albumin
cAMP	cyclic adenosine monophosphate
CBA	cytometric bead array
CCL	CC-chemokine ligands
CCR7	CC-chemokine receptor 7
CD	cluster of differentiation
CD40L	CD40 ligand
$CD56^{bright}$	$CD56^{bright}$ $CD16^{neg\text{-}dim}$ NK cells
$CD56^{dim}$	$CD56^{dim}$ $CD16^{bright}$ NK cells
CD95L	CD95 ligand
CFSE	carboxyfluorescein succinimidyl ester
CLI	critical limb ischemia
CLP	common lymphoid precursors
CMP	common myeloid precursors
ctr.	control
Dapi	4,6-Diamidin-2-Phenylindol Dihydrochlorid
DC	dendritic cells
DMSO	dimethylsulfoxid
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
Elispot	enzym-linked immunosorbent spot
ERK 1/2	extracellular-signal-regulated kinases 1/2
FACS	fluorescence-activated cell sorting
FCS	fetal calf serum
FcγRIII	Fc-gamma receptor III
FoxP3	forkhead box 3
FSC	forward scatter
G-CSF	granulocyte colony-stimulating factor

GM-CSF	granulocyte-macrophage colony-stimulating factor
GMP	good manufacturing practice
GvHD	graft-versus-host disease
HGF	hepatocyte growth factor
HLA	human leukocyte antigen
HSC	haematopoietic stem cells
ICAM1	intercellular adhesion molecule 1
IDO	Indoleamine-2,3-dioxygenase
IFN	interferon
Ig	immunoglobulin
IL	interleukin
ILC	innate lymphoid cells
IL-1Ra	interleukin-1 receptor antagonist
IL-10R	interleukin-10 receptor
i.m.	intramuscular
IP-10	interferon gamma-induced protein 10
IRF	interferon regulatory factor
ISCT	International Society for Cellular Therapy
iTreg	induced regulatory T cells
i.v.	intravenous
JNK	c-Jun N-terminal kinases
KIR	killer-cell immunoglobulin-like receptors
LPS	lipopolysaccharide
mAb	monoclonal antibody
MACS	Magnetic-activated cell sorting
M-CSF	macrophage colony-stimulating factor
MCP-1	monocyte chemotactic protein 1
mDC	myeloid dendritic cells
MHC	major histocompatibility complex
MFI	mean fluorescence intensity
MSC	mesenchymal stromal/stem cells
moDC	monocyte-derived dendritic cells
MS	multiple sclerosis
NCR	natural cytotoxicity receptors
NFκB	nuclear factor 'kappa-light-chain-enhancer' of activated B-cells
NIH	National Institutes of Health
NK cells	natural killer cells
NLR	NOD-like receptor
nTreg	naturally occurring regulatory T cells
n.d.	not detectable

n.s.	not significant
OCT	optimal cutting temperature
PAD	peripheral arterial disease
PAMP	pathogen-associated molecular patterns
PBMC	peripheral blood mononuclear cells
PBS	phosphate-buffered saline
pDC	plasmacytoid dendritic cells
PDL1	programmed death-ligand 1
PGE2	prostaglandin E2
PI	propidium iodide
PLX-PAD	placenta-derived mesenchymal-like adherent stromal cells
PRR	pattern-recognition receptors
R848	resiquimod
RAG	recombination activating gene
rel. to ctr.	relative to control
SD	standard deviation
SEB	staphylococcal enterotoxin B
SEM	standard error mean
sHLA-G5	soluble HLA-G5
SLE	systemic lupus erythematosus
SOP	standard operating procedure
SRY	sex-determining region Y
SSC	side scatter
SSEA-4	stage-specific embryonic antigen-4
STAT	signal transducer and activator of transcription
TCR	T cell receptor
$TcPO_2$	transcutaneous oxygen pressure
Td	dead K562 target cells
TGF	transforming growth factor
Th1	type 1 helper T cells
Th2	type 2 helper T cells
Th17	type 17 helper T cells
TNF	tumour necrosis factor
TLR	toll like receptor
Treg	regulatory T cells
TSDR	Treg specific demethylation region
VAS	visual analogue scale
VCAM	vascular cell adhesion molecule
VEGF	vascular endothelial growth factor

1. INTRODUCTION

1.1. The therapeutic potential of mesenchymal stromal/ stem cells (MSC)

1.1.1. Characterization and isolation of MSC

More than 40 years ago, Friedenstein et al. described a heterogeneous population of multipotent progenitor cells with a typical fibroblastoid phenotype [1]. These cells can differentiate *in vitro* into various cells of the mesodermal lineage, for instance adipocytes, chondroblasts and osteoblasts and have been named mesenchymal stromal/stem cells (MSC).

Since MSC lack specific and unique markers, the International Society for Cellular Therapy (ISCT) proposed three definition criteria in 2006 [2]. Firstly, MSC are characterised by a set of surface markers. Unfortunately, no lineage specific markers exist making multiparameter staining for inclusion/exclusion necessary. On the one hand, they express the surface markers cluster of differentiation (CD) 73, CD90 and CD105 (≥95%). On the other hand, they are negative for the haematopoietic lineage markers CD45, CD34, CD14 or CD11b, and CD19 or CD79α (≤2%). Moreover, MSC lack expression of the endothelial marker CD31 and costimulatory molecules like CD80, CD86 and CD40 (≤2%). Secondly, MSC adhere to plastic and thirdly, they differentiate under standard *in vitro* culture conditions into osteoblasts, chondroblasts and adipocytes. According to these definition criteria, MSC can be isolated from many different tissues, such as bone marrow, adipose tissue, umbilical cord, placenta or dental pulp. However, they represent only a very small percentage of tissue cells. MSC present approximately one cell in 10^4-10^5 mononuclear cells in bone marrow and about one cell per 10^2-10^3 cells from lipoaspirate [3,4]. Cells with most but not all MSC properties are called “MSC-like cells”.

To date, the number of CD markers that can be analysed per tissue section is limited because multiplex analysis of rare cells is difficult. Depending on the antibody combination and the microscope used, it is possible to analyse approximately five biomarkers at the same slide. Therefore, it was not possible to stain human MSC in tissue samples so far. It is not known if MSC can be detected *in vivo* by using the same

set of surface markers that is used for their identification and characterisation after *in vitro* expansion.

1.1.2. MSC used as cell therapy

Although their *in vivo* differentiation capacity has not clearly been proven, MSC are well known for their capacity to directly (by stimulating tissue repair processes and vascularization) and indirectly (for instance by immunomodulation) support endogenous tissue regeneration [5,6]. Therefore, MSC became promising candidates for regenerative therapies in a range of acute or chronic ischemic tissue injuries to immune-mediated pathogenic processes [7]. Moreover, MSC have been shown to be low immunogenic, which would allow application across major histocompatibility complex (MHC) barriers [5]. In addition, they display potent anti-inflammatory and immunomodulatory properties, while little is known about their mechanism of immune escape or immunomodulation.

At the time of writing this thesis, worldwide 261 ongoing clinical studies using MSC were listed on the website www.clinicaltrials.gov, which is maintained by the United States National Institutes of Health (NIH). These trials target a broad field of indications ranging from immunological problems, such as multiple sclerosis (MS), Crohn's Disease or kidney transplantation linked with subsequent graft-versus-host disease (GvHD), to regenerative scopes, for instance osteoarthritis, spinal cord injury or ischemic heart failure.

In the relevant literature, one can find an enormous amount of preclinical as well as clinical trials investigating a broad range of indications. *Regeneration:* On the one hand, MSC are used to improve acute as well as chronic injuries. The angiogenic and antioxidative benefits of MSC are investigated in preclinical as well as clinical studies as therapy option for myocardial infarction or acute kidney injury [8,9,10,11]. It has been shown in different animal models that MSC can improve bone fracture healing as well as muscle regeneration [12,13]. Beside acute injuries, MSC are also considered as therapy option for chronic ischemic injuries, for instance to treat critical limb ischemia (CLI) [14,15]. Additionally, MSC are investigated for the improvement of microvascular and secondary complications of diabetes mellitus [16]. Interestingly, it has recently been

shown that autologous MSC preserved the β-cell function in patients with newly diagnosed diabetes mellitus type 1 [17]. *Immunomodulation:* On the other hand, the immunomodulatory capacities of MSC might be exploited in order to treat different immunological disorders. It has been reported that allogeneic MHC-mismatched MSC prolong engraftment of skin transplants in a baboon model [18]. Many researchers consider MSC as a treatment option to reduce GvHD and facilitate the engraftment of haematopoietic stem cell (HSC) transplantations [19,20,21]. Additionally, the immunosuppressive as well as the regenerative potential of MSC makes them an interesting approach being explored for the treatment of MS [22,23]. Furthermore, MSC were also used to treat inflammatory bowel/ Crohn's disease [24,25,26].

In this work, patient samples from an open-label phase I/IIa dose-escalation study with 15 CLI patients have been analysed. CLI represents the natural end stage of peripheral arterial disease (PAD), which is a chronic disease that progressively restricts the blood flow in the limbs as a result of atherosclerosis, diabetes, or other inflammatory processes. About 1% of the population older than 50 years in the United States suffers from CLI, a rate that is approximately doubled in people aged >70 years [27]. CLI patients have a one-year amputation rate of about 30% and a mortality rate of 25% [28]. The estimated cost for treating CLI in the United States alone is $10 to $20 billion per year [27]. CLI is frequently associated with other clinical problems, for example (e.g.) hypertension, cardiovascular diseases or obesity. Commonly, the severity of PAD is categorized using the Fontaine classification, through which CLI falls into Fontaine grade III (pain at rest) and IV (tissue necrosis). MSC have been shown to significantly increase viability, migration and tube formation of endothelial cells, mainly through secretion of soluble factors that stimulate vasculogenesis and angiogenesis, for instance vascular endothelial growth factor (VEGF) and hepatocyte growth factor (HGF) [29]. Moreover, human placenta-derived mesenchymal-like adherent stromal cells (PLX-PAD) support revascularization in a preclinical mouse model of hind limb ischemia [15]. Therefore, MSC and their relatives are promising candidates for the treatment of CLI. In a dose-escalation observational phase I/IIa clinical trial presented here, CLI patients received one intramuscular (i.m.) injection of allogeneic MHC-unmatched PLX-PAD cells into their affected limb and safety as well as immunological effects were addressed. While no

significant safety concerns could be observed during the twelve months follow-up, there were many indications for the efficacy and an improvement of symptoms, presented by enhanced blood flow (as measured by ankle-brachial index [ABI] index), pain score (as assessed by visual analogue scale [VAS]), transcutaneous oxygen pressure ($TcPO_2$) and quality of life assessment. Moreover, a high rate of amputation-free survival (85% versus 65% in historic controls in the course of one year) was observed. Importantly, no sensitization specific to allogeneic PLX-PAD cells could be detected. In contrast, a unique inflammatory response and immunomodulation without hints for general immunosuppression was induced. Investigating the immunomodulatory effects of PLX-PAD cells in this clinical trial was part of this doctoral thesis. In particular, it was analysed if patients developed type 1 helper T (Th1) cell responses specific for the applied allogeneic PLX-PAD cells *in vivo*.

1.1.3. Advantages of using autologous or allogeneic MSC

Most clinical trials have been performed with autologous MSC, but there are also reports for the application of allogeneic MHC-unmatched MSC [5,30]. A normal immunocompetent recipient would attack transplanted foreign tissue, a process referred to as alloimmunity and resulting in graft rejection. For this reason, the application of autologous MSC would be safer and should be more efficient than the usage of allogeneic cells. Though, it is important to keep in mind, that the preparation of autologous MSC is time-consuming and needs complex logistics and regulatory requirements [31]. For clinical use, MSC have to be isolated under good manufactoring practice (GMP) conditions. Especially for bone marrow-derived MSC (BM-MSC), it has been reported that the cell yield negatively correlates with the donor age [32]. Due to their low frequency in the origin tissue, they need to be expanded, which takes up to several weeks, depending on the donor and the required cell number. Yet, long term culture decreases the proliferation rate and differentiation potential [33]. Additionally, there are some reports showing that autologous MSC can display disease and age-related impairments [34,35,36]. For these reasons, it would be preferable to use MSC as an “off-the-shelf“ MHC-unmatched cell product. However, this is an immunological challenge and it must be ensured that MSC are not immunogenic themselves.

Alloimmunogenicity of MSC would cause several problems. Firstly, efficacy of the cell therapy might decrease due to rejection of the cells. Secondly, a repeated MSC application would probably even lead to increased alloimmunogenicity and finally, immune complexes might cause direct tissue damages, e.g. in the kidney. Up to now, there are several preclinical and clinical trials, which report no safety concerns using allogeneic MSC [37,38]. Nevertheless, in other studies MSC indeed caused alloimmunity and even stimulated graft rejection [39,40,41]. Therefore, the immunogenicity of allogeneic MSC should be addressed in more clinical trials and immunological data absolutely need to be included to a larger extent.

1.2. Initiation of an immune response

1.2.1. Overview of the innate and adaptive immune system

All living beings are permanently exposed to infectious agents. Even single cell bacteria developed systems to fight back virus infections. Multicellular animals dedicate cells or tissues to treat infections. The human immune system is an extremely complex and powerful network that can be roughly classified into the innate and the adaptive arm of immunity, determined by speed and specificity of the reaction. The innate immune system is activated within minutes, but with limited specificity and almost without memory formation. It is followed by the slower antigen specific defence of the adaptive arm of immunity, which can often provide lifelong protection against reinfection due to the development of immunological memory.

The innate immune system groups different components of the body's first line of defence, including outer barriers like skin and mucosal membranes, the complement system, which tags pathogens for destruction by other cells, and cells of the innate immune system. These comprise neutrophils, innate lymphoid cells (ILC), among them natural killer (NK) cells, as well as phagocytic and antigen presenting cells (APC), such as monocytes, macrophages or dendritic cells (DC). Innate immune cells do not express recombination activating gene (RAG)-dependent rearranged antigen receptors, but rapidly respond to cytokines and cellular ligands, produced in tissues after infection or injury, as well as to pathogen associated molecular patterns (PAMP). Interleukin (IL)-12, IL-15, IL-18, tumour necrosis factor (TNF) α and interferon (IFN) γ are cytokines that

activate innate immune cells. They are secreted by other innate immune cells, e.g. DC, or by lymphocytes, mainly T cells. The dogma that innate immunity is nonspecific has been changed when pattern-recognition receptors (PRR) were discovered [42]. These recognize conserved PAMP, like bacterial and fungal cell wall components or viral nucleic acids. The PRR consist of several receptor classes, among them are membrane-bound receptors, including the toll like receptors (TLR), and cytosolic receptors, such as NOD-like receptors (NLR) [43,44,45].

The adaptive immunity is highly pathogen-specific, can be activated by the innate immune system and consists of humoral as well as of cell-mediated immunity. B cells (humoral immunity) and T lymphocytes (cellular immunity) are the key players of this part of the immune response and ensure the development of an immunological memory.

T cells develop in the thymus and get activated by binding of their T cell receptor (TCR) to its specific antigen, which has to be presented as peptide by MHC class I or II molecules on the surface of APC [46]. T cells are divided into $CD8^+$ cytotoxic T cells and $CD4^+$ T helper cells. Cytotoxic T cells recognize peptides presented by MHC class I molecules. These peptides are generated from cytosolic proteins and derive mainly from intracellular pathogens. $CD8^+$ T cells kill virus-infected cells, tumour cells and foreign tissue directly by releasing cytotoxins. T helper cells recognize peptides in the context of MHC class II presentation. These are proteins that were mostly internalized from extracellular medium and processed in endosomes or lysosomes. $CD4^+$ T cells help other immune cells, for instance macrophages, B cells and cytotoxic T cells, to fulfil their effector functions (e.g. antibody production by B cells). T cell help is mainly mediated by cytokine release and expression of CD40 ligand (CD40L). The latter binds to CD40 expressed by APC, thereby facilitating cell-cell communication and promoting cytokine secretion by APC. Moreover, CD40L induces B cell maturation.

B cells develop in the bone marrow and their main effector function is to produce antibodies, though they can also serve as APC. They recognize their specific soluble antigen via the B cell receptor (BCR). Yet, B cells need to receive a second activating signal, which could be delivered either by the T cell-independent or the T cell-dependent pathway. There are two possibilities for T cell-independent activation: additional TLR

signalling or binding of highly repetitive structures on the surface of pathogens leading to BCR cross-linking between different B cells. The T cell-dependent pathway means that an APC primes a $CD4^+$ T helper cell by presenting a processed antigen. Presentation of the same peptide by a B cell to the already activated T helper cell, leads to the binding of CD40L (on T cells) and CD40 (on B cells) and the release of cytokines by the T cell, among them IL-2, IL-4 or IL-5, which induce B cell activation.

Our immune system must distinguish between "self" and "non-self". Usually, self-reactive lymphocytes are neutralized by clonal deletion, abortion and anergy [47,48] and many regulatory mechanisms among others regulatory T cells (Treg) and tolerogenic APC exist to prevent autoimmunity [49,50,51]. This enables our immune system to tolerate autologous tissue while it can recognize harmful pathogens and also foreign tissue even from the same species, a process termed alloimmunity. The recognition of alloantigen is mainly due to differences in MHC genes between donor and recipient and can be mediated by the innate immune system, T or B cells [52]. Alloimmunity is important when thinking about organ transplantations and cell therapies, such as MSC application.

Only the close collaboration between the innate and the adaptive immune system can protect us effectively against pathogens. The complex network of innate and adaptive immune cells involves cell-cell interactions as well as soluble factors that might lead to either activation or suppression of immune responses and could be bidirectional [53]. For instance, DC induce NK cell activation, while NK cells can kill immature DC, but can also increase maturation of DC. The outcome of this complex NK cell/DC crosstalk is dependent on the activation status of both players. Moreover, APC activate T cells, which in turn deliver B cell help and enhance APC maturation. In addition, NK cells are important for T cell stimulation, especially for priming of naïve T cells into Th1 cells.

1.2.2. Antigen presenting cells (APC)

APC serve as a bridge linking the innate and adaptive immunity, because their main function is to take up antigens and present them on their MHC to T cells [54]. An antigen can be any peptide that serves as target for the TCR. The spectrum includes proteins from viruses, bacteria, fungi or foreign tissue. Even autologous proteins can serve as an

antigen, but normally self-reactive T cells are removed. One has to distinguish non-professional and professional APC, mainly based on the presence of MHC class I or II molecules on their cell surface and the ability to provide costimulatory signals [46]. Human MHC molecules are also called human leukocytes antigens (HLA). All nucleated cells express MHC class I to present peptides of intracellular proteins on their cell surface. In this way, infected body cells can serve as non-professional APC and present foreign peptides on their MHC class I, which is recognized by cytotoxic T cells. MHC class II molecules are only expressed by professional APC, including macrophages, B cells, thymic epithelial cells and in particular DC. Professional APC are characterized by a pronounced ability to take up and process antigens and to present antigen fragments (epitopes) via MHC class II molecules to $CD4^+$ T helper cells [55]. In addition, professional APC can activate $CD8^+$ cytotoxic T cells by cross-presentation, which means presentation of extracellular antigens on MHC class I molecules. Moreover, professional APC can be localized to secondary lymphoid organs and upregulate the expression of costimulatory molecules after antigen encounter, which is required to activate naïve T cells.

DC are the most important professional APC and consist of a heterogeneous cell population. They are classified according to their origin, location and function. DC development is challenging to study in humans because of the difficult accessibility, but it is well understood in mice. Gene expression analysis showed a good similarity between mouse and human DC classifications [56]. Human DC subpopulations originate from $CD34^+$ HSC in the bone marrow and undergo differentiation through common myeloid precursors (CMP), but a proportion can also be generated from common lymphoid precursors (CLP) [57,58]. When DC leave the bone marrow, they complete their differentiation to immature migratory DC or DC that are resident in lymphoid tissues. In the peripheral blood, as the only readily available source in humans, migratory DC are classified into two main groups: the classical or myeloid DC (mDC) and plasmacytoid DC (pDC). The blood dendritic cell antigens (BDCA) 1-4 are used to identify DC subsets. BDCA2 (CD303 or CLEC4C) and BDCA4 (CD304 or Neuropilin) define pDC. mDC are further divided into two subpopulations based on their expression of BDCA1 (CD1c) or BDCA3 (CD141 or thrombomodulin) [56]. Monocytes are precursors for

macrophages and DC, but it is not entirely clear into which human DC subset they develop *in vivo* [56]. Nevertheless, there are more DC subsets in the tissue.

After antigen encounter, DC run through a process called maturation which enables them to recruit and to activate various other cells of both the innate and the adaptive immune system. One of the crucial steps during the maturation process of DC is the acquisition of CC-chemokine receptor 7 (CCR7), since it allows migration towards draining lymph nodes [59]. Among others, naïve T cells, $CD56^{bright}$ NK cells and mature DC express CCR7. The main ligands are the chemokines CC-chemokine ligands (CCL) 19 and CCL21. Mature DC secrete pro-inflammatory cytokines and chemokines that attract other effector cells, among them NK and T cells. DC-derived cytokines, such as IL-12, IL-15 and IL-18, are well known stimulators of NK cell activation. IFNγ secretion, cytotoxicity as well as proliferation of NK cells have been shown to be enhanced by DC [60]. In turn, NK cells can induce maturation of DC and among others enhance their IL-12 production. After antigen uptake and processing by DC, peptides are displayed on MHC class II molecules and presented to $CD4^+$ T helper cells. For the activation of naïve T cells, TCR signalling has to be accompanied by additional costimulatory signals, for instance CD80, CD86 and CD40, which are also provided by professional DC. The cytokine profile of DC determines the lineage commitment of T cells, resulting in the differentiation of naïve T cells into distinct lineages of memory and effector T helper cells [61], which will be described in more detail in section 1.2.4.

In summary, the three hallmarks of DC maturation are:

i. CCR7-dependent migration towards lymph nodes
ii. Secretion of pro-inflammatory cytokines and chemokines: recruitment and activation of NK and T cells as well as lineage decision for T cell effector subsets
iii. Antigen presentation to T cells and supply of costimulatory signals

1.2.2.1. Monocyte-derived DC (moDC)

Human blood monocytes are classified into $CD14^+$ $CD16^-$ classical, $CD14^+$ $CD16^+$ intermediate and $CD14^{low}$ $CD16^+$ non-classical monocytes [62]. Monocytes leave the bone marrow, stay one to three days in the bloodstream, then typically migrate into tissues and differentiate into macrophages or DC. Until now, it is not totally understood

which human DC subsets derive from monocytes *in vivo*. Several studies have demonstrated that monocytes do not differentiate into mDC and that they make up a relatively small proportion of the lymphoid organ DC in the steady state [63,64]. Yet, gene expression analysis suggests that dermal $CD14^+$ DC and intestinal $CD103^-$ $CD172a^+$ DC are related to monocytes and therefore might derive from monocytes [65,66]. In addition, $CD1c^+$, $CD1a^+$ and $CD14^+$ inflammatory DC have been found in inflammatory fluids and transcriptome analysis revealed that they originate from monocytes [67].

In vitro, monocytes can be differentiated in the presence of granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-4 into so called monocyte-derived DC (moDC) [68]. These have a typical dendritic morphology and lack CD14 expression. Upon maturation by different factors, such as lipopolysaccharide (LPS), CD40L or TNFα, these cells express high levels of MHC class I and II, CD40 as well as CD80/CD86 [69]. Mature moDC produce cytokines and chemokines, for instance IL-1, IL-6, IL-12 or interferon gamma-induced protein 10 (IP-10), and have a high capacity to stimulate T cells *in vitro* (Figure 1) [68,69]. In addition, it has been shown that *in vitro* generated moDC upregulate CCR7 expression upon maturation by a variety of stimulating agents, for instance LPS, prostaglandin E2 (PGE2) or IFNγ [70,71]. However, recently, it has been shown that the gene signatures of *in vitro* generated moDC are (comparable to the *in vivo* data) more close to inflammatory DC than to classical mDC [67,72].

1.2.2.2. Plasmacytoid DC (pDC)

pDC are the key effector cells in early antiviral immunity [73]. They express high levels of CD303 (BDCA2), CD304 (BDCA4) and CD123 (IL-3 Receptor), but only low amounts of CD1c and CD141, which distinguishes them from mDC [74]. In the steady state, pDC are characterized by a relatively low level of costimulatory molecules as well as MHC class I and II [58]. They display a strong expression of the virus-sensing TLR7 and 9, whose activation leads to strong secretion of type I IFN (mainly IFNα) and induction of cytotoxic functions in pDC and NK cells [73,75]. Stimulated pDC upregulate MHC class II, costimulatory molecules and the chemokine receptor CCR7 [76,77]. They become professional APC and are able to cross-present antigens to $CD8^+$ T cells and also to prime

NK cells as well as $CD4^+$ T cells (Figure 1) [75,78,79]. Due the high secretion of type I IFN, pDC even have been reported to induce IL-12-independent Th1 priming [80].

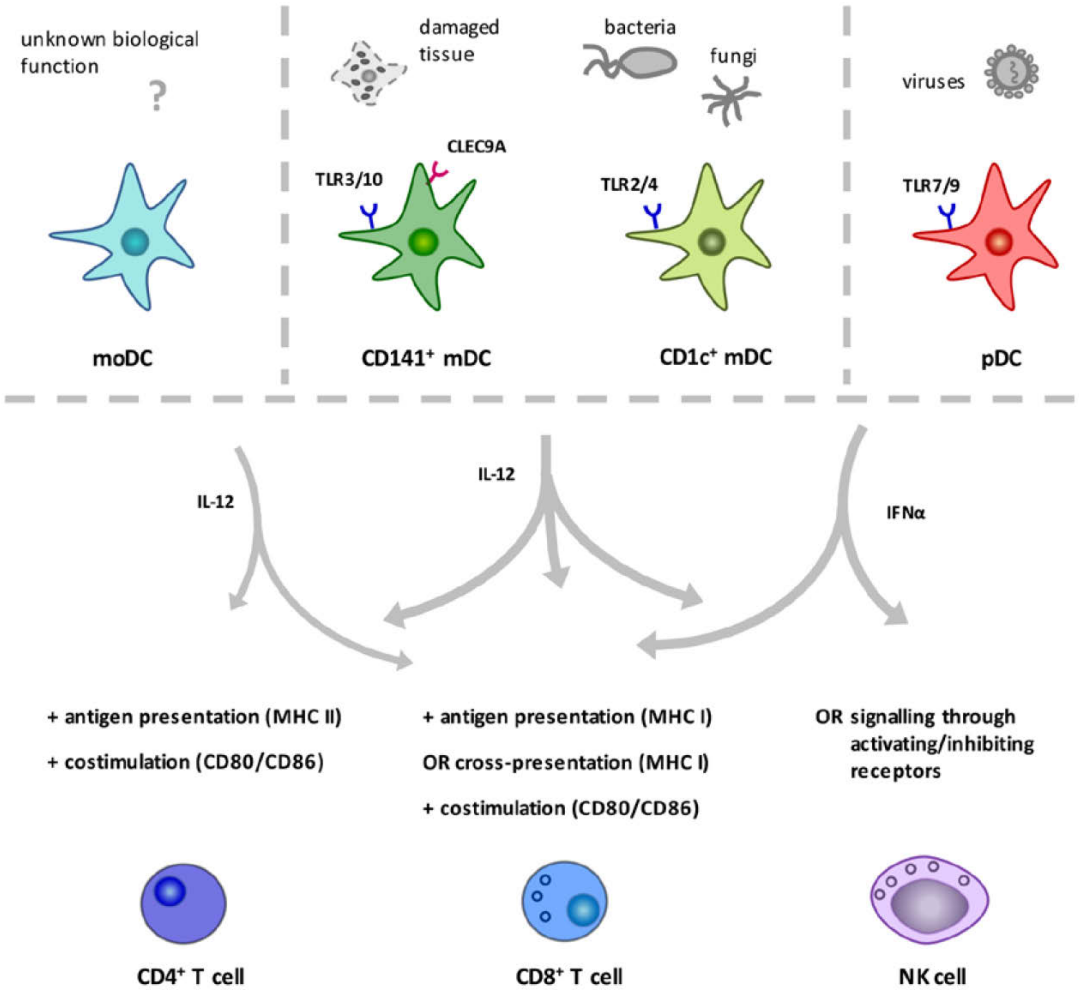

Figure 1: Biology of human DC subsets. The main activating mechanisms as well as priming of NK and T cells are shown for moDC, $CD141^+$ mDC, $CD1c^+$ mDC and pDC.

1.2.2.3. Myeloid DC (mDC)

mDC are specialized to sense fungi, bacteria and injured tissue [58]. They are able to capture environmental- and cell-derived antigens and display a high phagocytic capacity [73]. Immature mDC express high levels of MHC class II, but these are localized in late endosomes and lysosomes. Only after stimulation with microbial products or inflammatory mediators, endocytosis is suppressed and mDC acquire an increased ability for formation and accumulation of peptide/MHC class II complexes on their cell

surface. In addition, they upregulate surface expression of costimulatory molecules, such as CD80, CD86, CD40 or intercellular adhesion molecule 1 (ICAM1) [74]. Furthermore, TLR stimulation leads to high CCR7 expression [77].

Human mDC are classified into two subpopulations (Figure 1). The capacity for antigen uptake and presentation to $CD4^+$ and $CD8^+$ T cells is comparable in both subsets and higher than in moDC [81,82]. $CD141^+$ ($BDCA3^+$) mDC express TLR3 and 10 and make up 5-10% of the peripheral DC respectively [83]. In response to TLR3 signalling, they produce high amounts of chemokines and pro-inflammatory cytokines IL-12, IFNγ and IFNβ, leading to efficient Th1 priming and activation of NK and cytotoxic T cells (Figure 1) [73,84]. Due to expression of the C-type lectin CLEC9A, $CD141^+$ mDC can sense damaged tissue and necrotic cells [85,86]. In contrast, $CD1c^+$ ($BDCA1^+$) mDC express all TLR1-10, except for TLR9, which allows them to detect a wide range of bacteria and fungi [87]. In particular, they are characterized by high levels of TLR2 and 4. $CD1c^+$ mDC account for approximately 50% of peripheral blood DC [83]. Upon TLR stimulation, they secrete a broad range of pro-inflammatory cytokines, among them TNFα, IL-1β, IL-6 and IL-12 as well as the chemokine IP-10 [73,87]. Because of their secretome and their strong antigen processing capacity, $CD1c^+$ mDC are effective inducers for NK cell activation as well as Th1 and cytotoxic T cell responses (Figure 1) [73]. Both mDC subsets upregulate CCR7 expression when appropriately stimulated by PRR [77]. In this study, $CD1c^+$ mDC were used, since they can readily be isolated from human blood.

1.2.2.4. Tolerogenic DC

The term tolerogenic DC is not specific for a DC subset or restricted to the immature state of DC [88]. The main feature of tolerogenic DC is their low expression of costimulatory molecules like CD80, CD86 and CD40. Moreover, they express high levels of surface molecules involved in T cell inhibition, such as programmed death-ligand 1 (PDL1) and CD95 ligand (CD95L) [89]. Tolerogenic DC are capable for antigen presentation to T cells, but fail to provide sufficient costimulatory signals or even deliver coinhibitory signals. Thereby, they rather induce T cell apoptosis, anergy or the generation of Treg [90,91]. Moreover, tolerogenic DC secrete low levels of pro-inflammatory cytokines, including IL-12, but rather anti-inflammatory cytokines like

IL-10, which suppress immune responses [88]. Thymic DC are known to induce tolerance to self-antigens *in vivo* by clonal deletion of self-reactive $CD4^+$ $CD8^+$ thymocytes.

Due to their characteristics, the induction of tolerogenic DC might also be important when thinking about controlling alloimmunity, e.g. for MSC applications. It has been shown that tolerogenic DC can be generated *in vitro* through addition of different anti-inflammatory and immunosuppressive agents. Among them are IL-10, transforming growth factor (TGF) β1, HLA G and inducers of cyclic adenosine monophosphate (cAMP), for instance PGE2 or histamine [92]. Some of these factors have already been shown to play a role in the mechanism of DC modulation by MSC (see paragraph 1.3.2).

1.2.3. Natural Killer (NK) cell-mediated immunity

NK cells belong to the innate immune system and play an important role for the body's first line of defence, e.g. against virus infections and tumours. Their main effector functions are the release of cytotoxins in order to lyse target cells and the production of cytokines (e.g. IFNγ, TNFα) that mediate inflammation. NK cells express two types of surface receptors controlling their activity: activating and inhibitory receptors. The activation status is a balance between both signals. The main stimulating receptors are the natural cytotoxicity receptors (NCR, e.g. NKp46, NKp30, NKp44), CD16 (Fc-gamma receptor III, FcγRIII) and NKG2D [93]. Killer-cell immunoglobulin-like receptors (KIR) and CD94/NKG2A deliver the most important inhibitory signals [93]. Inhibitory receptors recognize self MHC class I molecules. Therefore, injection of allogeneic MSC might also lead to activation of recipient NK cells since the MSC might not deliver the respective inhibitory signals. Moreover, NK cells can also be activated by cytokines, among them IL-2, IL-12, IL-15 and IL-18 [94]. Apart from IL-2, which is mainly produced by T cells, these cytokines are secreted by DC. IL-12 and IL-18 induce IFNγ production and enhance cytotoxicity by NK cells, whereas IL-15 promotes NK cell proliferation [94]. Additionally, IFNα/β secreted by different DC types has been shown to enhance NK cell cytotoxicity [95]. Nevertheless, some authors also reported that surface receptor interactions were necessary for NK cell activation by DC [94].

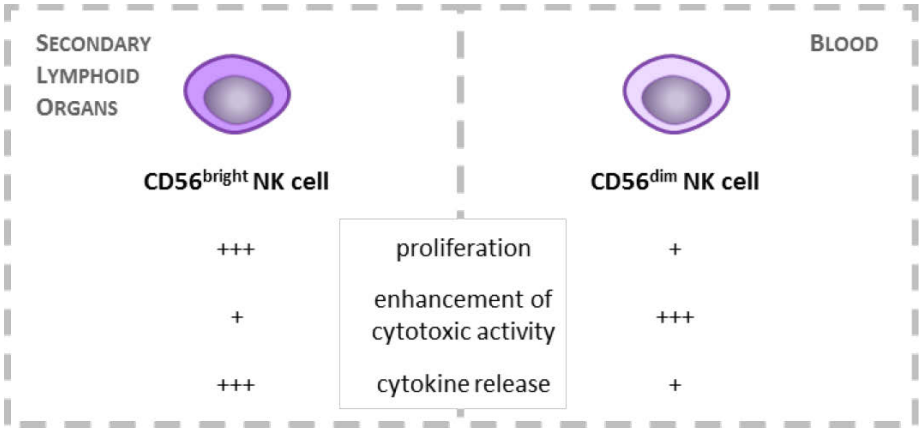

Figure 2: NK cell subsets. When activated by cytokines (e.g. provided by DC), $CD56^{bright}$ and $CD56^{dim}$ NK cells display different capacities for proliferation, cytokine release and enhancement of cytotoxicity.

Peripheral NK cells can be divided into subpopulations (Figure 2). The two major subsets are $CD56^{bright}$ $CD16^{neg\text{-}dim}$ ($CD56^{bright}$) and $CD56^{dim}$ $CD16^{bright}$ ($CD56^{dim}$) NK cells. They have different functional properties, which are associated with various receptor repertoires and distinct homing capacities [96]. The CCR7-expressing $CD56^{bright}$ cells are the majority of NK cells in secondary lymphoid organs and make up only about 10% of peripheral blood NK cells [97,98]. They are considered to be the main cytokine producers upon activation by cytokines [96,99]. $CD56^{bright}$ NK cells have a poor cytolytic activity and a high proliferative capacity (Figure 2). In contrast, $CD56^{dim}$ NK cells preferentially express chemokine receptors like CXCR1 that mediate migration towards inflamed peripheral tissues. Indeed, this subset makes up about 90% of peripheral blood NK cells. $CD56^{dim}$ NK cells have a higher cytotoxic activity, a lower proliferative capacity and secrete lower cytokine levels than the $CD56^{bright}$ compartment when stimulated by cytokines (Figure 2) [96,99]. However, it is now known that $CD56^{dim}$ NK cells produce high levels of mRNA for IFNγ and that they secrete large amounts of different cytokines upon stimulation by activating receptors [100,101]. $CD56^{dim}$ NK cells probably derive from the $CD56^{bright}$ compartment and represent a stage of more mature peripheral NK cells [99,102].

Upon activation, NK cells not only become cytolytic against target cells, but also release cytokines and chemokines that induce inflammatory responses and influence quality as well as strength of T cell responses [99]. Especially, NK cell-derived IFNγ is known to be important for Th1 priming of naïve T cells, since it increases the capacity of DC to produce IL-12 (see section 1.2.5) [103].

1.2.4. T helper cell-mediated immunity

T cells develop in the thymus and their main characteristic is the recognition of peptide-loaded MHC molecules on professional or non-professional APC by the TCR on their cell surface (see section 1.2.2). During the course of an immune response, they differentiate from naïve towards effector and memory T cells.

There exist several T cell subsets with distinct functions. The first classification is used according to the expression of the TCR co-receptors CD4 and CD8 [46]. T helper cells express CD4, which recognizes MHC class II molecules on the surface of APC and amplifies the TCR signal. The CD8 molecule, expressed by cytotoxic T cells, recognizes the MHC class I, leading to a stronger binding of the target cell to the TCR [46]. The T helper cell population can differentiate from naïve $CD4^+$ T cells into various types of memory and effector T cell subsets (Figure 3), classified according to their cytokine profile, pattern of surface molecules and functionality. T helper cells are divided into at least four subpopulations (Figure 3): the type 1, 2 or 17 helper T cells (Th1, Th2 and Th17 respectively) and Treg. Nevertheless, there are even more subsets.

IFNγ is the signature cytokine of the Th1 lineage (Figure 3), but these cells also produce high amounts of IL-2, TNFα and TNFβ [104]. Th1 cell-derived IFNγ triggers e.g. macrophages to clear intracellular pathogens and induces immunoglobulin (Ig) G2 production by B cells [105]. Th1 cell activation is linked to many organ-specific autoimmune diseases [106]. Lineage commitment of naïve T cells towards the Th1 compartment has been shown to be IL-12- and IFNγ-dependent, while IL-4 should be absent [104,107]. The key transcription factors determining Th1 differentiation are T-bet and signal transducer and activator of transcription (STAT) 4 [108]. STAT4 is one of the main signal transducers for IL-12 and therefore important for the amplification of Th1 responses [109]. It also directly induces IFNγ production [106].

In contrast, Th2 cells mainly secrete IL-4, but also IL-5, IL-10 and IL-13 (Figure 3) [107]. Th2 cell-derived cytokines control the activation of eosinophils, basophils, mast cells and the mucosal epithelia [105]. IL-4 regulates B cell class switch to IgE, being important for the clearance of extracellular pathogens [107]. Th2 cells have been shown to be responsible for different allergic inflammatory diseases and asthma [106]. The

generation of Th2 cells needs signalling of IL-4 and IL-2, with Gata3 (for IL-4) and STAT5 (for IL-2) being the key transcription factors [108,110].

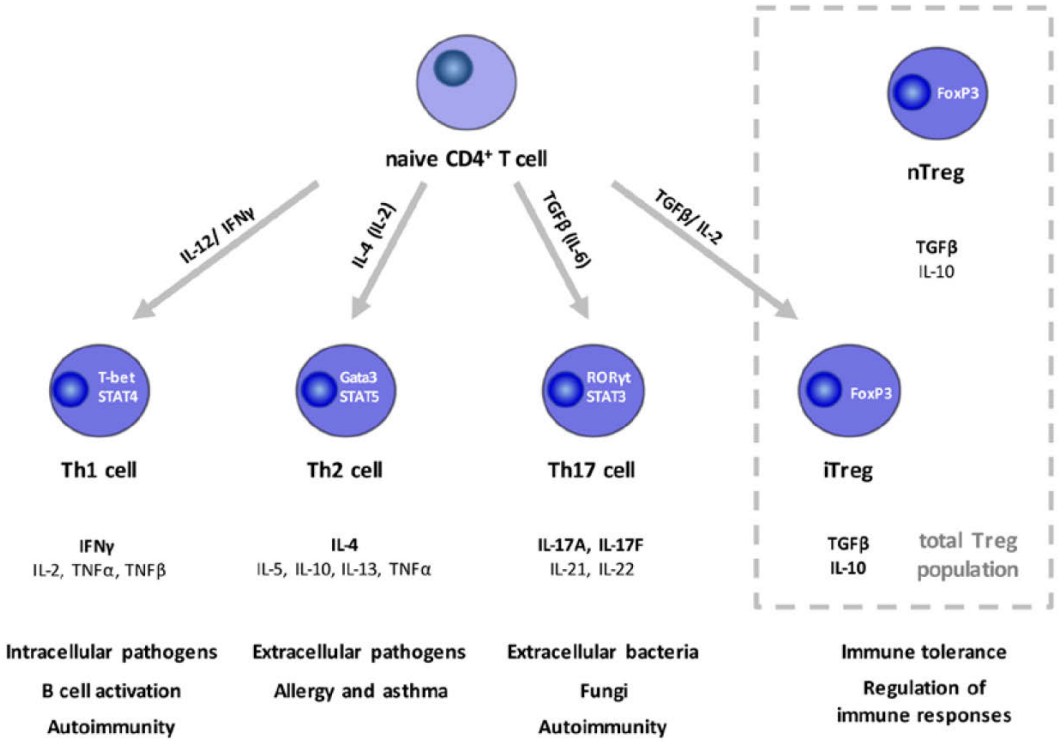

Figure 3: Overview of the most important T helper cell populations in the periphery. Cytokines and transcription factors determining the fate of $CD4^+$ T cell subsets as well as their signature cytokines and main functions are shown.

The name for Th17 cells originates from their signature cytokines IL-17A and IL-17F, though they also produce high amounts of IL-21 and IL-22 (Figure 3) [107]. Th17 cells are involved in many autoimmune and chronic diseases, but are also important for the clearance of extracellular bacteria and fungi [111]. Their differentiation is mainly mediated by TGFβ and IL-6 [105] with RORγt and STAT3 being the key transcription factors [107].

Treg represent the fourth major $CD4^+$ subset (Figure 3). Their main role is to shut down T cell responses and to suppress autoreactive T cells that escaped the negative selection in the thymus [112]. They are investigated as a treatment option for autoimmune diseases and for the prevention of allograft-rejection. Some of their suppressive mechanisms depend on cell-cell contact, while others are mediated through cytokines, including TGFβ and IL-10 [106]. Treg are divided into two subpopulations. Naturally

occurring Treg (nTreg) derive from the thymus, while generation of induced Treg (iTreg) from naïve $CD4^+$ T cells is mediated by IL-2 and TGFβ signalling and CD28 costimulation [107,113]. The lineage-specific transcription factor that determines the fate for Treg lineage is forkhead box 3 (FoxP3) [112]. The Treg specific demethylation region (TSDR) on nTreg is completely demethylated, thus their FoxP3 expression is stable. However, TSDR of iTreg are only partly demethylated [114], so they have low FoxP3 expression. Additionally, nTreg mainly prevent autoimmunity to keep immune tolerance, while iTreg are believed to mainly control chronic allergic inflammation especially in the gut and to be one of the important barriers for tumour cells [114].

1.2.5. Crosstalk between DC, T cells and NK cells

The complex network between DC, NK and T cells mainly takes place in secondary lymphoid organs or inflamed peripheral tissues (Figure 4) [94,115]. Activation of naïve T cells leads to clonal expansion and differentiation into effector/memory T cells and is controlled by several checkpoints [116,117,118,119]:

i. DC migration to draining lymph nodes
ii. Chemokine release by DC leading to recruitment of NK, T and B cells
iii. Secretion of pro-inflammatory cytokines by DC
iv. NK cell activation including IFNγ secretion, being essential for Th1 priming
v. Antigen presentation and costimulation by DC

When DC catch an antigen in the periphery, they mature, leading to CCR7-dependent migration towards draining lymph nodes, antigen presentation by MHC class II molecules, expression of costimulatory molecules and secretion of pro-inflammatory cytokines and chemokines. At the beginning of an immune response and before stimulation of naïve T cells, DC encounter $CD56^{bright}$ NK cells in the lymph nodes [120,121,122]. DC activate NK cells, whereas NK cells in turn can kill immature DC, but can also enhance their maturation. The final result of the complex interplay between NK cells and DC depends on the activation status of both players. NK cells respond very efficiently to DC-derived cytokines, mainly IL-12, by proliferation, enhanced cytolytic ability and IFNγ production (see paragraph 1.2.3) [123,124,125]. In turn, IFNγ secreted

by NK cells enhances DC maturation and thereby IL-12 secretion, which leads to increased Th1 priming [103,120,126,127]. Moreover, NK cell-derived IFNγ directly affects differentiation of $CD4^+$ T cells by activating STAT1, which up-regulates T-bet, the transcription factor for Th1 lineage commitment inducing IFNγ production and expression of IL-12 receptor [128]. In this way, binding of DC-derived IL-12 to T cells can be increased. Thus, the interplay between IFNγ and IL-12 leads to full Th1 differentiation [106].

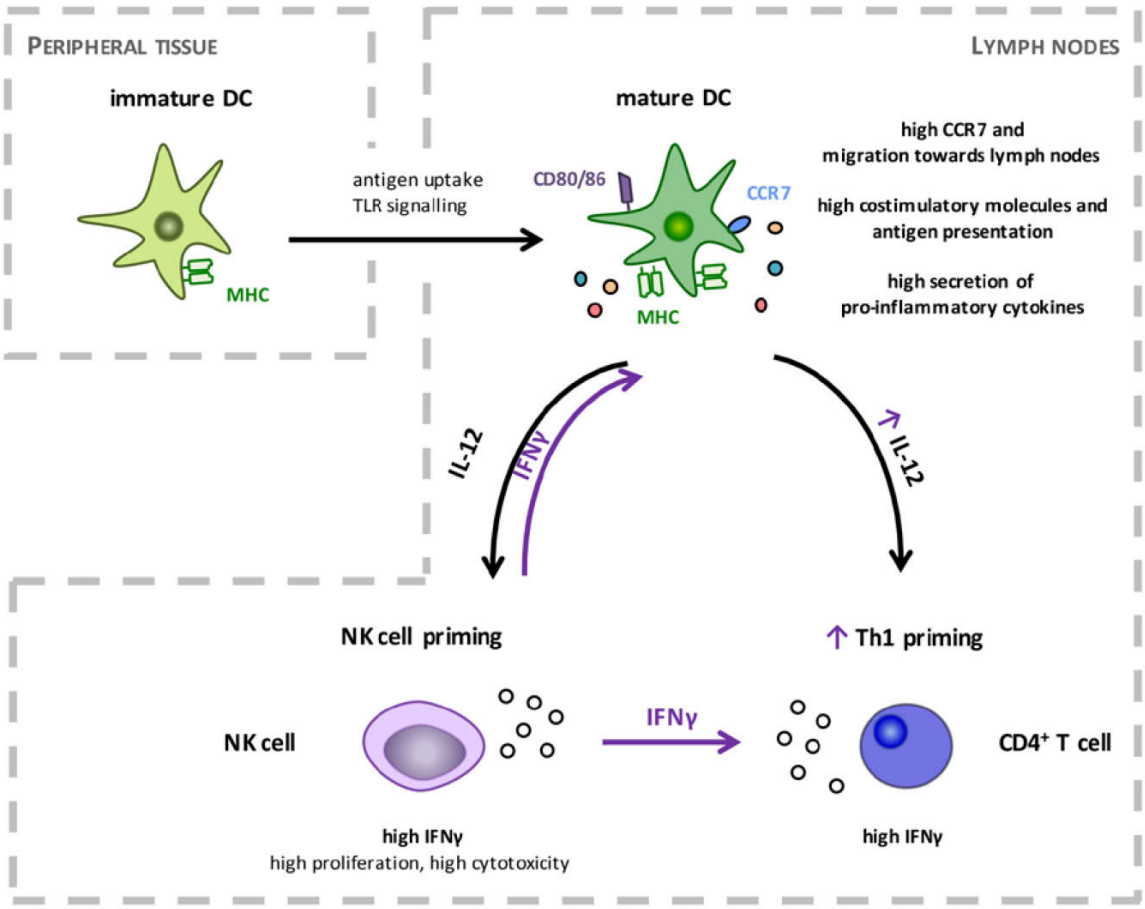

Figure 4: Crosstalk between DC, T cells and NK cells. In the periphery, immature DC encounter antigens. The maturation process is characterized by three main features: up-regulation of costimulatory molecules, secretion of pro-inflammatory cytokines and acquisition of CCR7 expression, leading to migration towards draining lymph nodes. There, DC-derived IL-12 activates NK and naïve T cells. NK cell-derived IFNγ increases IL-12 production by DC, leading to higher Th1 priming of T cells, and directly induces Th1 priming in $CD4^+$ T cells.

1.3. Immunogenicity and immunomodulation of MSC

1.3.1. General aspects of immunogenicity and immunosuppression of MSC

There is hope to use MSC as treatment for a broad range of acute or chronic ischemic tissue injuries and immunological disorders. Many preclinical as well as clinical trials using allogeneic MSC have not observed severe side effects indicating that MSC do not trigger an immune response by themselves [37,38]. However, others indeed have observed alloimmunity to MSC *in vitro* and *in vivo* [39,41].

The best route of administration for MSC application remains questionable. It has been shown that after local administration (for instance i.m.) the cells persist at the injection site for two to four weeks [129,130]. On the contrary, intravenous (i.v.) injection leads to large sequestration of cells in the lung within hours, but their systemic effects can be detected for several weeks [131,132]. Moreover, there are contradictory reports regarding the *in vivo* engraftment of MSC. Most data show that MSC engraft and differentiate *in vivo* only with very poor efficiency [133,134], but this has not been observed in human studies. In summary, the general consensus is that MSC serve as a biological drug delivery system by secreting soluble factors.

1.3.2. MSC induce tolerogenic DC

It is well known that MSC inhibit the *in vitro* differentiation of human $CD14^+$ monocytes or haematopoietic $CD34^+$ precursors into DC [135,136]. DC derived from both precursors normally acquire high CD1a expression and lack CD14 expression. In contrast, they have not gained CD1a expression and have kept high levels of CD14 when MSC were present during the differentiation process [136]. These DC also express lower levels of HLA-DR and of costimulatory molecules CD80, CD83, CD86 and CD40 after maturation [39,137,138]. In contrast to these clear data on the influence of MSC on DC differentiation *in vitro*, there are contradictory reports regarding the question if MSC also interfere with the progression from immature to mature DC. For instance, in the study of Jiang et al. MSC suppress LPS-induced maturation of moDC *in vitro*, while data generated by Spaggiari and colleagues do not support this hypothesis [137,139].

DC maturation also goes along with acquisition of CCR7 expression, mediating migration into draining lymph nodes. Yet, it has been shown that murine MSC suppress CCR7 acquisition by bone marrow-derived murine DC *in vitro* [140,141]. Likewise, Chiesa et al. have demonstrated in a mouse model that i.v. injected MSC reduce the number of DC expressing CCR7 and CD49dβ1, the latter being another molecule involved in DC migration towards lymph nodes [132]. This results in less migration towards draining lymph nodes and reduced priming of antigen-specific T cells. To date, it has not been investigated if MSC influence the migration of human DC.

Some studies also have investigated if MSC interfere with cytokine secretion by DC. Indeed, it has been shown that mouse and human moDC generated from monocytes or $CD34^+$ HSC in the presence of MSC secrete less pro-inflammatory cytokines TNFα and IL-12 *in vitro* [132,138,142]. Interestingly, Spaggiari et al. have shown that human MSC only inhibit IL-12 production by moDC when they have been present during the differentiation process, while there is no effect when MSC were only present during *in vitro* maturation [137]. This suggests that MSC do not directly affect maturation and thereby DC's ability to produce cytokines, but the altered cytokine production rather has to be seen as a consequence of the disturbed DC generation. Yet, the situation is probably more complex *in vivo*. In immune and nonimmune tissues, MSC would not only encounter DC precursors but also already differentiated immature DC. Therefore, it is necessary to understand whether MSC interfere with both, differentiation as well as maturation of DC. In contrast to consistent data on IL-12 production, there are contradictory *in vitro* data regarding the IL-10 level in MSC/moDC co-cultures compared to moDC alone. Two studies using human moDC have shown an increased IL-10 secretion in the presence of MSC [138,142], while another one has reported less IL-10 in co-cultures of murine DC and MSC [132]. Nevertheless, these studies only have investigated few key cytokines, but there are data missing for a broader picture on the influence of MSC on the secretome of DC.

So far, there is no unique mechanism known for immunomodulation by MSC. Different factors and mechanistic details have been reported to participate in inhibition of DC differentiation by MSC. The majority of publications show that different soluble factors play a role and that cell-cell contact between MSC and DC is not necessary [143]. PGE2,

a MSC product, has been shown to be an important mediator for inhibition of DC differentiation [137,144]. Two other studies have shown the involvement of IL-10, IL-6 and macrophage colony-stimulating factor (M-CSF), but only when MSC were present during early DC differentiation [139,145]. Nevertheless not only soluble factors seem to cause the inhibitory effects of MSC. At least a partial role has been proposed for contact-dependent activation of Jagged-2 or Notch signalling, too [138,146]. Also, MSC downregulate key molecules downstream of TLR4 signalling [132], resulting for example in a reduced IL-12 production.

In summary, there is a large body of evidence that MSC induce the generation of tolerogenic DC when they are present during the differentiation process. These DC are characterized by a low expression of costimulatory molecules and a rather anti-inflammatory cytokine profile (see paragraph 1.2.2.4). This is strengthened by numerous reports showing an impaired ability of DC that have been generated in the presence of MSC to induce allogeneic T cell proliferation *in vitro* [136].

The DC source is the main limitation of most of the publications about MSC-DC interactions so far. Due to the low accessibility of DC in the body, most studies have been performed with *in vitro* differentiated DC that were either generated from murine bone marrow or from human monocytes. These DC have two major limitations. Firstly, it has been shown for *in vitro* as well as *in vivo* generated moDC that they rather resemble inflammatory than conventional DC (see section 1.2.2.1) [56,63,72]. This means that moDC do not represent an adequate DC subpopulation to simulate the *in vivo* situation for MSC applications. Secondly, MSC will not only encounter DC precursors *in vivo* but most likely will interact with already differentiated immature DC. As discussed earlier, the effect of MSC on immature DC is much less understood. So far, Aggarwal et al. have published the only known study, showing that $CD1c^+$ mDC secrete less TNFα in the presence of MSC [147].

1.3.3. MSC inhibit effector functions of NK cells but are not resistant to NK cell mediated killing

MSC express low or intermediate levels of MHC class I molecules [148] and are often applied from HLA-unmatched donors. In addition, MSC even express different ligands for

activating NK cell receptors, for instance NKG2D or NKp30 ligands [136]. For these reasons, autologous as well as allogeneic MSC should be targets for NK cell mediated lysis, particularly in an inflammatory environment, which delivers activating signals for NK cells [5]. Two interesting studies support the idea that NK cells kill autologous and allogeneic MSC to the same extent *in vitro* [149,150], but NK cells have to be preactivated by cytokines, e.g. IL-2 or IL-15 [149,151]. On the contrary, MSC suppress cytokine-induced NK cell proliferation, cytotoxicity and cytokine production, among them IFNγ, TNFα and IL-10 [151]. Moreover, NK cells display a diminished expression of activation markers (such as NKp44, NKp30 or NKG2D) in the presence of MSC [149,152].

Transwell experiments have revealed that MSC suppress proliferation, expression of activation markers and cytokine production of NK cells by soluble factors, whereas they require direct cell-cell contact to diminish the lytic potential of NK cells [151,152]. Blocking of Indoleamine-2,3-dioxygenase (IDO) activity restores NK cell proliferation in the presence of MSC. Decrease of cytotoxicity has been shown to be dependent on PGE2 and soluble HLA-G5 (sHLA-G5) [152,153]. Until now, most available data derive from *in vitro* studies, which investigated the direct impact of MSC on NK cell activation. All of them did not take into account NK cell activation by DC, which especially occurs in inflamed peripheral tissues or secondary lymphoid organs.

1.3.4. MSC modulate the functionality of T cells

The suppressive effects of MSC on T cell functionality have initially been described over a decade ago [154]. Since then, suppression of $CD4^+$ T helper lymphocytes as well as $CD8^+$ cytotoxic T cells has been reported [155]. Most of these studies have been performed using peripheral blood mononuclear cells (PBMC) for mixed lymphocyte reactions. In this case, T cell suppression by MSC could either happen directly by inhibiting T cells or indirectly by modifying APC. Indeed, some authors have shown that removal of monocytes from PBMC leads to a lower inhibitory rate of MSC [156,157], suggesting that MSC rather modulate the functionality of APC then T cells themselves. However, different studies have indicated that immunomodulation and especially T cell suppression by MSC need preactivation by IFNγ [158,159]. It has also been shown that isolated Th1 cells secrete less IFNγ in the presence of MSC *in vitro*, whereas Th2 cells

produce higher levels of IL-4, indicating a shift from a pro-inflammatory Th1 towards an anti-inflammatory Th2 response [147]. Moreover, MSC have been reported to increase the Treg number and to suppress generation of Th17 cells *in vitro* [160,161]. Higher levels of typical Th2 cytokines, such as IL-4, IL-10 or IL-13, and a decreased IFNγ production by Th1 cells in combination with an increased Treg frequency have also been reported in several animal models [162,163,164,165]. Enhanced Treg generation in the presence of MSC could be associated with protection from different immune system related diseases and even alloimmunity [166]. Interestingly, Treg depletion in a mouse model of allergic airway inflammation abrogated the suppressive impact [167].

Mechanistically, mainly soluble factors have been shown to mediate the suppressive effects on T cells, but at least a partial role for cell-cell contact dependent mechanisms has been reported, too [5]. PGE2 and IDO seem to be the major soluble mediators for inhibition of T cell proliferation [168,169]. IDO is not constantly expressed by MSC, it rather needs preactivation by IFNγ [170]. Nevertheless, cell-cell contact dependent mechanisms, for instance mediated by the negative costimulatory molecule B7-H4 or the PDL1 pathway, seem to play at least a partial role for T cell suppression by MSC [158,160].

1.3.5. Do MSC interfere with the crosstalk between DC, NK cells and T cells?

The current studies only consider the isolated impact of MSC on either DC, NK or T cells, not taking into account the complex and important interactions between the three cell types (see section 1.2.5 and Figure 5). It takes place in secondary lymphoid organs or inflamed peripheral tissues and is necessary to initiate immune responses.

However, MSC may interfere with the different parts and players of this communication network, especially in the *in vivo* situation where injected MSC could meet all of them. For instance, MSC might intervene with the maturation of immature (tissue) DC, which are probably one of the first cell types that they encounter after injection. This may lead to a reduced or altered ability for priming of NK and T cells by DC. Moreover, MSC might directly inhibit NK cell functionality with regard of DC activation or killing and their influence on Th1 priming by releasing IFNγ. Finally, MSC might directly inhibit or modify

functionality of T cells. Therefore, the investigation of this important crosstalk definitely deserves more attention in future studies.

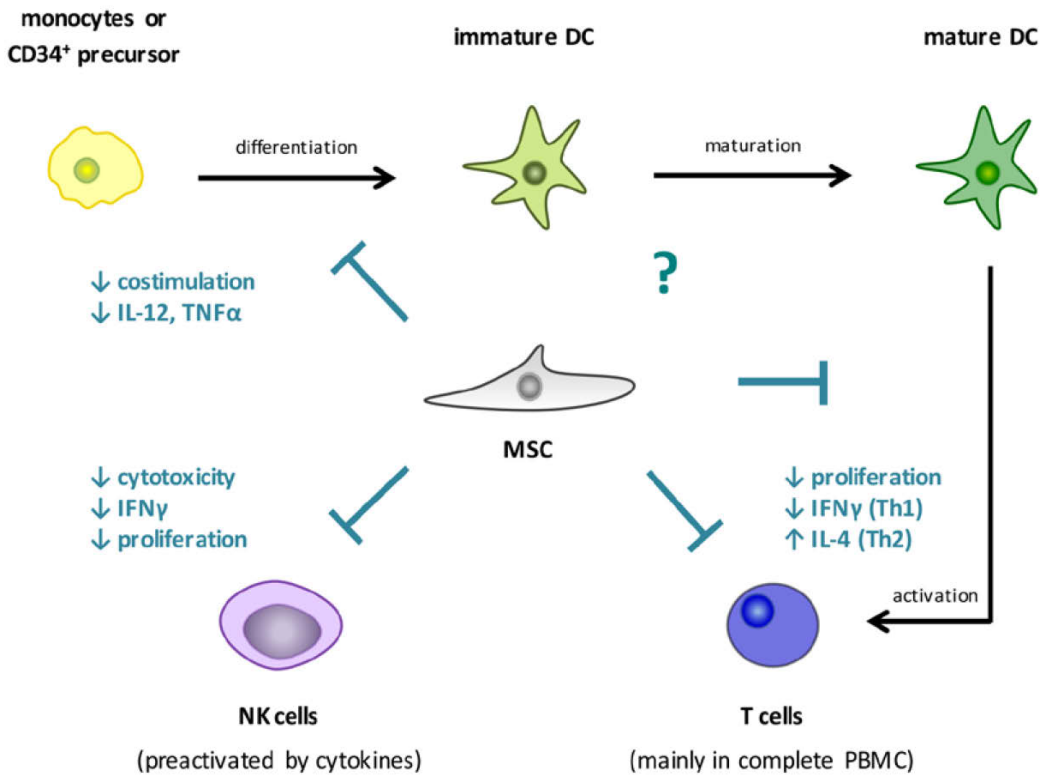

Figure 5: MSC have direct effects on DC, NK cells and T cells. MSC interfere with the differentiation of monocytes or $CD34^+$ precursors into DC, while it is not clear if they also affect DC maturation. In addition, they suppress T cell functionality and cytokine-mediated NK cell activation.

2. AIMS OF THIS WORK

There can be no doubt that MSC have a significant capacity to modulate immune responses. However, the underlying mechanisms behind their immunomodulatory properties are only poorly understood. Moreover, the reports on alloimmunogenicity demonstrate some inconsistency that might be related to different MSC subsets. By revealing the mechanisms behind, it might be possible to develop tests for pretesting their immunomodulatory capacity.

Most studies investigating the effect of MSC on antigen-presenting cells used *in vitro* generated DC either derived from human monocytes or $CD34^+$ precursors or murine bone marrow since human DC are difficult to access in sufficient numbers. *In vivo*, monocytes differentiate into a subpopulation of macrophages and inflammatory DC, but they do not give rise to classical mDC limiting their usability as model for the analysis of MSC-DC interaction [56,57,63,72]. Therefore, our study was designed to analyse how MSC influence *ex vivo* isolated physiologically more relevant human DC populations.

Since recent studies neglected to investigate if MSC interfere with the complex and important crosstalk between DC, NK and T cells, this question was also addressed here.

Beside the numerous reports on preclinical data, mostly *in vitro*, there are no adequate immunological *in vivo* data available, particularly from patients. Furthermore, it should be taken into account that patients might be presensitized to different alloantigens. Hence, there is a high need for immunological data from clinical trials using allogeneic MSC.

Therefore, three main topics have been investigated in this study (Figure 6):

I. Which impact do BM-MSC have on the maturation of *in vitro* generated moDC in comparison to *ex vivo* isolated pDC and mDC?
   a. Expression of maturation markers
   b. CCR7-dependent migration
   c. Secretion of cytokines, chemokines and growth factors

II. Do BM-MSC interfere with the crosstalk between DC, NK and T cells by influencing the maturation process of DC?

a. Ability of mDC to activate NK cells
b. Molecular mechanism for the observed effects
c. Ability of MSC-licensed mDC and moDC to activate T cells

III. Do CLI patients, who were treated with MSC-like HLA-unmatched PLX-PAD cells in a phase I/IIa clinical trial, develop T cell alloreactivity towards the MSC product?
   a. Memory T cell responses specific for the applied PLX-PAD or third party donor cells by performing IFNγ Enzym-linked immunosorbent spot (Elispot) with patient samples at different time points before and after PLX-PAD administration *in vivo*

IV. Can MSC be detected *in vivo* by the same set of CD markers that is used for *in vitro* characterisation?
   a. Staining of human placenta cryosections for $CD73^+$ $CD90^+$ $CD105^+$ $CD45^-$ $CD34^-$ $CD14^-$ $CD19^-$ cells by multiplex-immunohistology (chipcytometry)

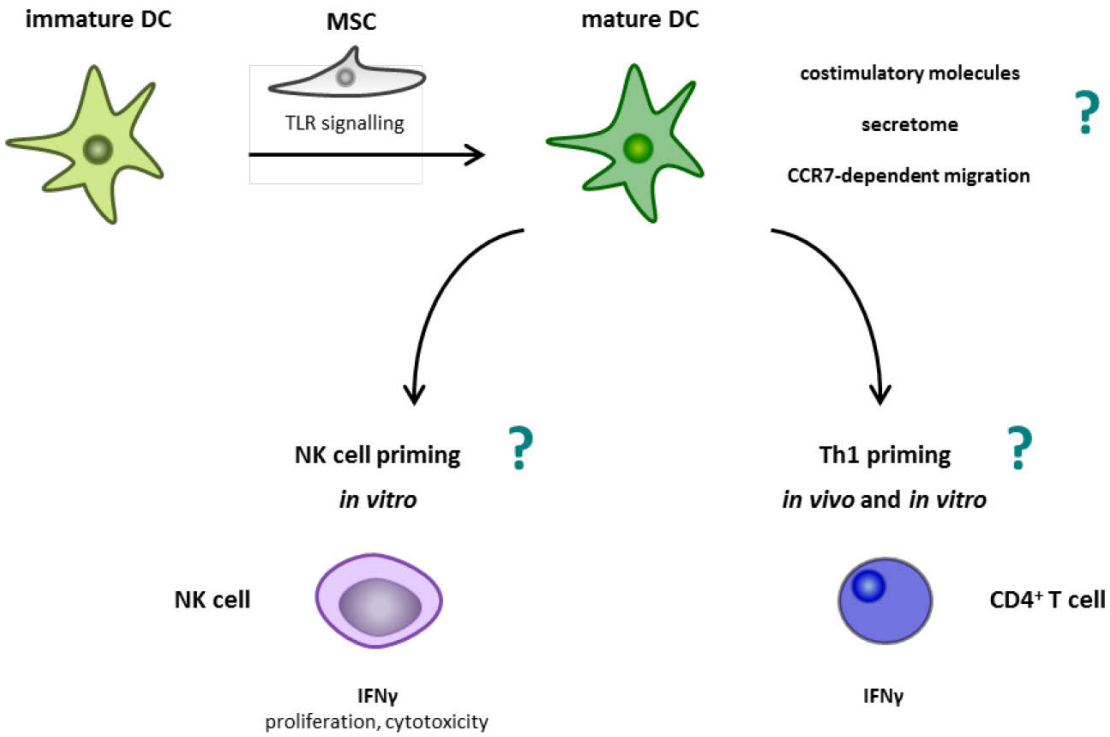

Figure 6: Aims of this work. The main goal of this thesis was to understand how MSC interfere with the communication network between DC, NK and T cells by interfering with the DC maturation process.

3. MATERIAL AND METHODS

3.1. Materials

3.1.1. Subjects

Peripheral blood leukocyte concentrates of healthy adults from the German Red Cross (DRK) were used to isolate immune cells. The relevant institutional review boards approved the study and all subjects gave their written informed consent according to the Declaration of Helsinki.

3.1.2. Clinical study design

The phase I/IIa safety study was approved by the Paul-Ehrlich-Institut and the institutional review boards (EudraCT number 2008-003711-13, study number PLX-PAD1202-01). It was performed at the Franziskus Hospital in Berlin, Germany and included 15 patients suffering from CLI, Fountaine class III/IV, Rutherford Category 4/5. They received a single treatment with PLX-PAD cells at three different dose levels (low dose $175x10^6$ cells [n=3], intermediate dose $300x10^6$ cells [n=6] or high dose $600x10^6$ cells [n=6]) at 50 i.m. injection sites above/below the knee of the most afflicted limb within less than 20min. PLX-PAD cells were generated from five different placentas. HLA-A, B, DRB and DQB typing revealed three to six mismatches between PLX-PAD donor cells and recipient. Blood was drawn before, three days, one week and four weeks after PLX-PAD treatment for immunological analyses. An IFNγ Elispot was performed to detect memory/effector T cells specific for PLX-PAD cells (see section 3.2.15) [171].

3.1.3. Equipment and devices

Table 1: List of main equipment and devices.

Equipment or device	Company
Cleanbench	Thermo Fisher
Quadro MACS Separation Unit	Miltenyi Biotech
Octo MACS Separation Unit	Miltenyi Biotech
MS Columns	Miltenyi Biotech
LS Columns	Miltenyi Biotech

Equipment or device	Company
Pre-Separation Filters	Miltenyi Biotech
Waterbath	SUB
Pipettes	Eppendorf Research
Cell Counter CASY	Innovatis
CO_2 Incubator	Sanyo
Heraeus Multifuge X1R (Centrifuge)	Thermo Scientific
Heraeus Multifuge 3SR+ (Centrifuge)	Thermo Scientific
Heraeus Biofuge fresco (Centrifuge)	Thermo Fisher
Microtest U-Bottom (tissue-culture treated 96 well round bottom plate)	BD Falcon
TC-Plate 96 well, Standard, F (tissue-culture treated 96 well flat bottom plate)	Sarstedt
Multiwell 12 well (tissue-culture treated 12 well plate)	BD Falcon BD
HTS Transwell-96 Permeable Support, 1.0µm Pore Polyester Membrane (3380)	Corning
HTS Transwell-96 Permeable Support, 0.4µm Pore Polycarbonate Membrane (3381)	Corning
HTS Transwell-96 Permeable Support, 5.0µm Pore Polycarbonate Membrane (3388)	Corning
HTS Transwell-96 Receiver Plate, tissue culture treated (3382)	Corning
Polyester Membrane Transwell-Clear Inserts for 24 well plates (3470)	Corning
Tissue Culture Dish	BD Falcon
Tissue Culture Flask 75cm^2	BD Falcon
Tissue Culture Flask 175cm^2	BD Falcon
ZellSafe_C chips	Zellkraftwerk
ZellSafe_T chips	Zellkraftwerk
24x46mm cover slips	R. Langenbrinck
Flow Cytometer, LSR II	BD Biosciences

Equipment or device	Company
Flow Cytometer, FACSCalibur	BD Biosciences
FACS (Cell Sorter), FACSAria II	BD Biosciences
Titramax 101 (orbital shaker)	Heidolph
Bio-Plex Pro II Wash Station	Bio-Rad
Bio-Plex 200 System (Multiplex reader)	Bio-Rad
ImmunoSpot (Elispot reader)	Cellular Technology Limited (C.T.L.)
FACS Diva Software	BD Biosciences
FlowJo	Tree Star
Bio-Plex Manager software	Bio-Rad
FCAP Array v3	BD Biosciences
GraphPad Prism 5	GraphPad Software

3.1.4. Chemicals and reagents

Table 2: List of chemicals and reagents.

Chemical or reagent	Company
Penicillin	Biochrom (now Merck Millipore)
Streptomycin	Biochrom (now Merck Millipore)
L-Alanyl-L-Glutamine	Biochrom (now Merck Millipore)
basic fibroblast growth factor (bFGF)	PeproTech
ethylenediaminetetraacetic acid (EDTA)	Promega
bovine serum albumin (BSA)	Sigma-Aldrich
Lipopolysaccharide (LPS)	Sigma-Aldrich
Resiquimod (R848)	Alexis
CpG-A (ODN 2216)	InvivoGen
CpG-B (ODN 2006)	InvivoGen
Staphylococcal enterotoxin B (SEB)	Sigma-Aldrich
IL-3	R&D Systems
IL-4	R&D Systems
GM-CSF	R&D Systems
CCL21	R&D Systems
GolgiStop (containing Monensin)	BD Biosciences

Chemical or reagent	Company
Brefeldin A (5mg/ml in 70% ethanol)	Sigma-Aldrich
37% Formaldehyde	Sigma-Aldrich
Carboxyfluorescein succinimidyl ester (CFSE, 5mM in Dimethylsulfoxid [DMSO])	Invitrogen, Molecular Probes
FcR Blocking Reagent, human	Miltenyi Biotech
4,6-Diamidin-2-Phenylindol Dihydrochlorid (Dapi, 300µg/ml in water)	Invitrogen, Molecular Probes
Propidium iodide (PI)	Sigma-Aldrich
LIVE/DEAD Fixable Aqua dead cell stain kit	Invitrogen, Molecular Probes
CountBright Absolute Counting Beads	Invitrogen, Molecular Probes

3.1.5. Sera, media, buffers and solutions

Table 3: List of sera, media, buffer and solutions.

Medium or buffer	Company
Hepes	Biochrom (now Merck Millipore)
Human AB serum	Lonza
Fetal calf serum (FCS)	Biochrom (now Merck Millipore)
RPMI-1640 medium	Biochrom (now Merck Millipore)
DMEM low glucose medium	Biochrom (now Merck Millipore)
Biocoll	Biochrom (now Merck Millipore)
Phosphate-buffered saline (PBS)	gibco
Ampuwa (sterile water)	Fresinius Kabi
Buffer EL, Erythrocyte lysis buffer	Qiagen
Perm/Wash Buffer 10x	BD Biosciences
Zellkraftwerk wash buffer	Zellkraftwerk
Zellkraftwerk fixation buffer	Zellkraftwerk
Zellkraftwerk storage buffer	Zellkraftwerk
optimal cutting temperature (OCT) compound	VWR

Heat inactivation of serum

Human AB serum or fetal calf serum (FCS) were heat inactivated before usage to inactivate complement. After complete thawing, the serum was incubated in a prewarmed water bath at 56°C for 30min. 5ml aliquots were stored at -20°C until further usage.

Complete RPMI-1640

A 500ml bottle of RPMI-1640 medium was supplemented with 100U/ml penicillin, 0.1mg/ml streptomycin, 2mM L-Glutamine and 20mM Hepes and stored at 4°C. For experimental usage, 5ml of human AB serum was given to 45ml of RPMI-1640 (final concentration of 10% AB serum).

Complete DMEM

10% FCS, 100U/ml penicillin, 0.1mg/ml streptomycin, 2mM L-Glutamine, 20mM Hepes and 2ng/ml bFGF were added to a 500ml bottle of DMEM medium and stored at 4°C until further usage.

Magnetic-activated cell separation (MACS) buffer

A 500ml bottle of PBS was supplemented with 2 mM ethylenediaminetetraacetic acid (EDTA) and 0.5% FCS.

Fluorescence-activated cell sorting (FACS) buffer

0.5% FCS was given to a 500ml bottle of PBS.

Fixation buffer

In a 50ml Falcon tube, PBS buffer was complemented with 2% formaldehyde. The solution was used up to one month.

1x Perm/Wash buffer

In a 50ml Falcon tube, one part of 10x stock solution Perm/Wash buffer was diluted with nine parts of water.

Zellkraftwerk relaxation buffer

In a 10ml Falcon tube, PBS buffer was supplemented with 0.5% bovine serum albumin (BSA) and 2mM EDTA.

3.1.6. Kits

Table 4: List of kits for cell isolation and determination of cytokine concentrations.

Name of kit	Company
CD4 MicroBeads, Human	Miltenyi Biotech
CD14 MicroBeads, Human	Miltenyi Biotech
CD56 MicroBeads, Human	Miltenyi Biotech
Anti-Fitc MicroBeads	Miltenyi Biotech
Anti-PE MicroBeads	Miltenyi Biotech
Human Pan T cell Isolation Kit	Miltenyi Biotech
Bio-Plex Pro Human Cytokine Grp I Panel 27-Plex	Bio-Rad
Bio-Plex Pro Human Cytokine Group II (Customized: IL-12p40, IL-18, MCP-3, VCAM-1) 3-plex	Bio-Rad
Human IFN alpha 1 ELISA Ready-SET-Go! RSG	eBioscience
Human Inflammatory Cytokine Kit	BD Biosciences
Human Th1/Th2/Th17 Cytokine Kit	BD Biosciences
IFNγ Elispot Assay Kit	AID

3.1.7. Antibodies

Table 5: List of antibodies against human antigens.

Antibody (Clone)	Conjugate	Company
CD1c (AD5-8E7)	Fitc	Miltenyi Biotech
CD3 (UCHT1)	APC	BD Biosciences
CD3 (UCHT1)	PacB	BD Biosciences
CD3 (HIT3a)	PE	BD Biosciences
CD4 (RPA-T4)	APC Cy7	BioLegend
CD4 (RPA-T4)	A700	BioLegend

Antibody (Clone)	Conjugate	Company
CD4 (SK3)	PE Cy7	BD Biosciences
CD4 (SK3)	Percp	BD Biosciences
CD4 (SFCI12T4D11)	ECD	Beckman coulter
CD14 (M5E2)	Percp Cy5.5	BD Biosciences
CD14 (M5E2)	PE Cy7	BD Biosciences
CD14 (M5E2)	V421	BioLegend
CD19 (HIB19)	PE Cy7	BioLegend
CD19 (HIB19)	V421	BioLegend
CD40 (5C3)	APC-Cy7	BioLegend
CD40 (G28.5)	Fitc	BioLegend
CD45 (5B1)	APC	Miltenyi Biotech
CD45 (5B1)	Fitc	Miltenyi Biotech
CD45 (5B1)	PE	Miltenyi Biotech
CD45RA (HI100)	A488	BioLegend
CD45RO (UCHL1)	PE	BioLegend
CD56 (AF12-7H3)	PE	Miltenyi Biotech
CD56 (B159)	PE Cy7	BD Biosciences
CD56 (HCD56)	PE Cy7	BioLegend
CD62L (DREG-56)	APC-eFluor 780	eBioscience
CD80 (2D10)	Fitc	BioLegend
CD80 (2D10)	PE	BioLegend
CD83 (HB15e)	Percp Cy5.5	BioLegend
CD86 (IT2.2)	Biotin	BioLegend
CD107a (H4A3)	APC	BioLegend
CD107a (H4A3)	Fitc	BD Biosciences
CD304 (AD5-17F6)	PE	Miltenyi
CCR7 (TG8/CCR7)	A647	BioLegend
CCR7 (G043H7)	A647	BioLegend
HLA-DR (L243)	A700	BioLegend
IFNγ (B27)	A700	BD Biosciences
Streptavidin	PacO	Invitrogen
Streptavidin	PE	Invitrogen

Table 6: List of neutralizing antibodies.

Antibody (Clone)	Company
anti-human IL-10 (JES3-19F1)	BD Biosciences
anti-human IL-10R (3F9)	BioLegend
anti-human IL-1Ra (10309)	R&D Systems
anti-human IL-1Ra (polyclonal)	R&D Systems
anti-human IL-6 (polyclonal)	R&D Systems
anti-human CD28 (CD28.2)	BD Biosciences

3.1.8. MSC and cell lines

Table 7: Cell lines.

Cell type	Company
BM-MSC (Cat.No 7500)	ScienCell
PLX-PAD	Pluristem Therapeutics
K562	ATCC

3.2. Methods

3.2.1. BM-MSC and PLX-PAD expansion

Third party human BM-MSC were purchased at ScienCell and expanded in complete DMEM low glucose complemented with 10% FCS, 100U/ml penicillin, 0.1mg/ml streptomycin, 2mM L-Glutamine, 20mM Hepes and 2ng/ml bFGF until passage three to five. Cells were aliquoted and frozen in liquid nitrogen until further usage. For *in vitro* experiments, BM-MSC were thawed and seeded one day in advance in complete RPMI-1640 to allow attachment.

PLX-PAD cells were provided by Pluristem Therapeutics (Haifa, Israel). The PLX-PAD production process has been described by Ramot et al. [130]. In short, adherent cells were grown in two-dimensional culture flasks, before being further expanded on a fibrous carrier material in a dedicated three-dimensional bioreactor system. Aliquoted PLX-PAD cells were shipped frozen and stored in liquid nitrogen until further usage. For *in vitro* experiments, cells were thawed and seeded one day in advance in complete

RPMI-1640 to allow attachment of PLX-PAD. For clinical application, cells were thawed and injected immediately i.m. without further cultivation steps.

3.2.2. Isolation of peripheral blood mononuclear cells (PBMC)

The term PBMC includes all blood cells containing one nucleus, among them T cells, B cells, NK cells, DC and monocytes. PBMC were separated from erythrocytes, granulocytes and plasma in whole blood using density gradient centrifugation with Biocoll (Figure 7).

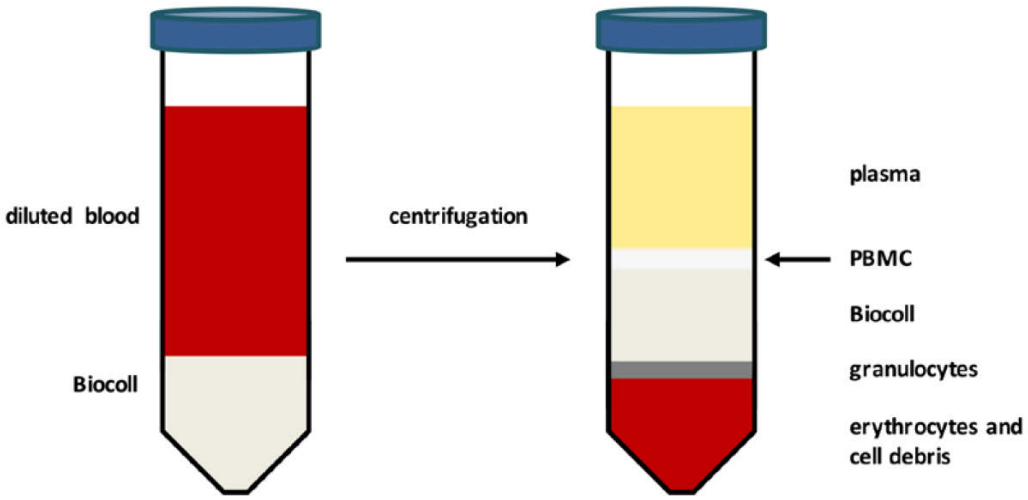

Figure 7: Isolation of PBMC from peripheral blood. Blood was diluted 1:1 with MACS buffer and carefully layered onto Biocoll. During gradient centrifugation, PBMC were enriched in the interphase between plasma and Biocoll. Granulocytes, erythrocytes and cell debris were pelleted.

The blood from one buffy coat (around 80-100ml) was diluted 1:1 with MACS buffer. 35ml of blood/buffer solution was carefully transferred onto 15ml Biocoll each and centrifuged at 2,000rpm for 20min at room temperature without acceleration and brake. Due to the different density of individual cell populations, PBMC were enriched in the interface between plasma (upper layer containing thrombocytes) and Biocoll (Figure 7). Granulocytes and erythrocytes were pelleted under the Biocoll. The PBMC ring was transferred into a new falcon tube and washed with 50ml MACS buffer to remove rests of Biocoll (centrifugation at 1,500rpm, 4°C, 10min). The supernatant was decanted and all pellets combined in 50ml MACS buffer before final centrifugation to remove thrombocytes (900rpm, 4°C, 15min). When a lot of erythrocytes remained after this

step, they were lysed by applying 40ml of Erythrocyte lysis buffer and 10ml of MACS buffer onto the cell pellet. PBMC were counted with CASY cell counter according to the manual.

3.2.3. Magnetic-activated cell sorting (MACS)

PBMC can be sorted by magnetic-activated cell sorting (MACS) to obtain different cell populations. This method was developed by Milteyni Biotech and uses small magnetic nanoparticles, so called MicroBeads, which are coupled to antibodies specific for different cell surface antigens. Thereby, target cell populations can be magnetically labelled and separated in a magnetic field from unlabelled cells (Figure 8). It is possible to either enrich (positive selection) or to deplete (negative selection) a specific cell population.

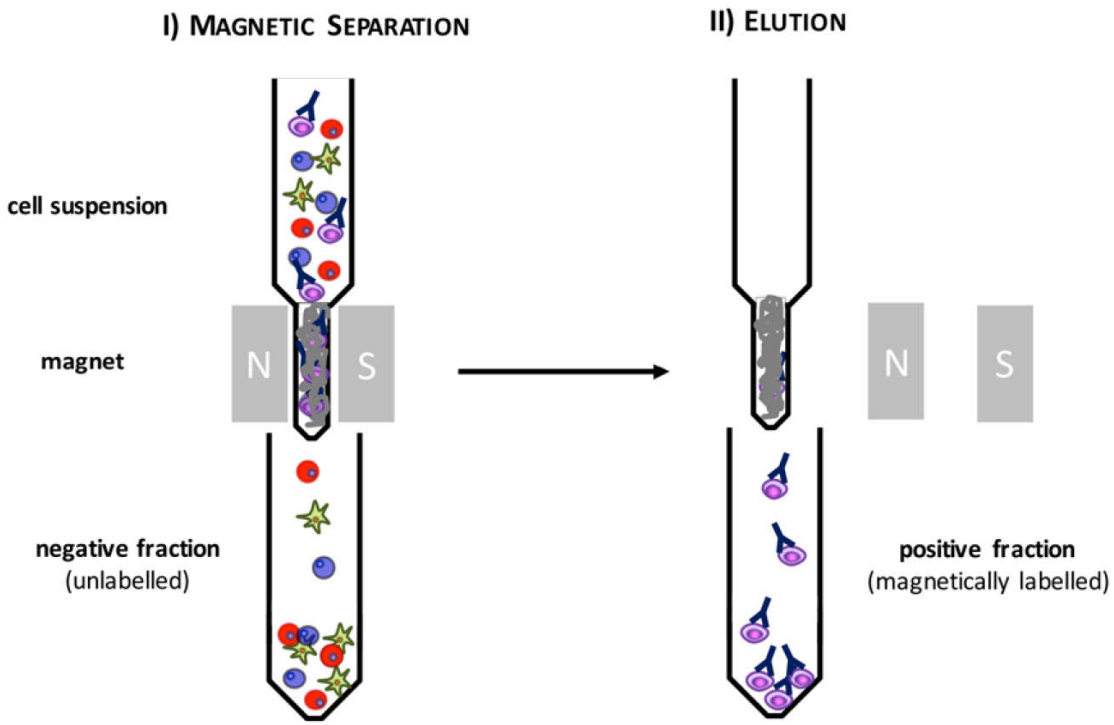

Figure 8: Principle of MACS. In a first step, a cell suspension containing magnetically labelled cells is added onto a MACS column. The flow through contains the unlabelled cells (negative fraction) while magnetically labelled cells are retained within the column and are eluted in a second step after removal from the magnetic field (positive fraction).

Table 8: Magnetic labelling of different cell populations for MACS. Antibodies, beads and protocols for labelling of $CD14^+$, $CD1c^+$, $CD304^+$, $CD56^+$, $CD3^+$ and $CD3^+$ cells.

	Antibody labelling		**Magnetic labelling**		
	Antibody	**Incubation**	**MACS MicroBeads**	**Incubation**	**Columns**
CD14	not applicable		200µl CD14 MicroBeads + 800µl MACS buffer	15min, 4°C	2 LS
CD1c	250µl CD1c Fitc (directly on the pellet)	10min, 4°C	250µl anti-Fitc MicroBeads (directly on the pellet)	15min, 4°C	1 LS
CD304	250µl CD304 PE (directly on the pellet)	10min, 4°C	250µl anti-PE MicroBeads (directly on the pellet)	15min, 4°C	1 LS
CD56	not applicable		250µl CD56 MicroBeads + 1ml MACS buffer	15min, 4°C	1 LS
CD4	not applicable		CD4 MicroBeads	15min, 4°C	2 LS
CD3	10µl Pan Biotin Antibody Cocktail/ $1x10^7$ cells	10min, 4°C	10µl Pan MicroBeads/ $1x10^7$ cells	15min, 4°C	1 MS

The different protocols used in this work are summarized in Table 8. Monocytes were separated by CD14 MicroBeads. CD4 MicroBeads were used for the enrichment of $CD4^+$ T helper cells from $CD14^-$ PBMC. mDC were enriched using anti-CD1c Fitc and anti-Fitc MicroBeads. NK cells were enriched from $CD1c^-$ PBMC using CD56 MicroBeads. The Pan T cell Isolation Kit was used to enrich untouched total $CD3^+$ T cells from $CD1c^-$ $CD56^-$ PBMC. The labelled sample was added onto a MACS column (see Table 8) and placed in a magnetic MACS Separator (Figure 8). Unlabelled cells passed through the column (flow through) while magnetically labelled cells were retained within the column. The column was washed three times with 3ml MACS buffer and removed from the magnetic field. The labelled target cell fraction was eluted twice with 5ml MACS buffer from the column. In the case of CD3 separation, the unlabelled flow through contained the T cells. Target cells were counted with CASY cell counter according to the manual.

3.2.4. Flow cytometry based methods

Flow cytometry is a laser-supported method to analyse light scattering and fluorescence of single cells that pass successively through a flow cell. Cells are labelled with fluorochrome-conjugated monoclonal antibodies (mAb) specific for one target molecule.

Each fluorochrome emits light with a specific wavelength when excited by a laser beam. Detectors pick up the light scattered by the cells and emitted by the excited fluorochromes. The forward scatter (FSC) is placed in line with the light beam and correlates with the cell size or volume. The side scatter (SSC) is positioned perpendicular to the light beam and defines the granularity of the cells. The other detectors collect emitted fluorescent light by a complex system of optical filters and mirrors. The signals are visualized on a screen. Flow cytometry is used extensively to study expression of many different intra- and extracellular markers, allowing for instance analysis of immune cell populations. Moreover, a heterogeneous mixture of cells can be sorted into its subpopulations, a technique named fluorescence-activated cell sorting (FACS).

3.2.4.1. Immunofluorescence staining of cell surface antigens

A list of all mAb used during this work is shown in paragraph 3.1.7, Table 5. Suitable concentrations for all mAb were determined beforehand. To stain surface molecules, samples were incubated with premixed antibody cocktails in 50µl FACS buffer at 4°C for 10min. Only CCR7 was stained in the presence of FcR Blocking Reagent at 37°C for 15min. Subsequently, unbound antibodies were washed out with 1ml FACS buffer. To avoid fluorochrome bleaching, exposition to light was minimized. Dapi or PI was added to the cell suspensions directly ahead of flow cytometric analysis. Both reagents incorporate in the DNA after passing the damaged membrane of dead cells, thereby allowing exclusion of dead cells from analysis.

3.2.4.2. Staining of intracellular antigens

For intracellular staining of IFNγ, which is normally secreted by the cells, Brefeldin A was added to the stimulation culture at different time points before harvesting (see section 3.2.13.3 and 3.2.14.2). To allow antibodies to pass the cell membrane, cells need to be fixed and permeabilized beforehand. Before intracellular staining for IFNγ, samples were first stained for surface markers and dead cells in 50µl PBS without serum at room temperature for 20min. LIVE/DEAD a fixable dye was used for the staining of dead cells. After one washing step with 1ml PBS without serum, cells were fixed with 2% formaldehyde in PBS buffer at room temperature for 10min. The samples were washed

with 1ml 1x Perm/Wash buffer before intracellular staining at room temperature for 20min. After a final washing step with 1x Perm/Wash buffer, cells were analysed at the LSR II.

3.2.4.3. Labelling of cells with carboxyfluorescein succinimidyl ester (CFSE)

Carboxyfluorescein succinimidyl ester (CFSE) is a fluorescent dye that passes cell membranes and covalently couples to intracellular molecules. Due to its fluorescence halving following each cell division, CFSE can be used to track lymphocyte proliferation. Up to $5x10^6$ cells per 0.5ml were stained in 1-5µM CFSE in PBS without serum at room temperature for 4min in the dark. The reaction was stopped with complete medium. T or NK cells were cultured with DC for five to six days before analysing the proliferation rate by flow cytometry, as measured by CFSE dilution.

3.2.4.4. Fluorescence-activated cell sorting (FACS)

Using FACS, a heterogeneous mixture of cells can be sorted into its subpopulations. The principles of a cell sorter and flow cytometer resemble each other (see paragraph 3.2.4). By setting gates in the FACS Diva Software, it is possible to define particular target cell populations. The measured cells reach the nozzle point, where they are divided into single drops (up to $2x10^5$ drops/sec). The target cells are tagged by an electrical charge and thereby can be directed through an electrostatic field into a collection tube.

Table 9: Antibody panels for cell sorting.

Target population	Antibody panel	Gating
$CD1c^+$ mDC	CD1c Fitc (from MACS), CD14 PE Cy7, CD19 PE-Cy7, Dapi	$Dapi^-$ $CD14^-$ $CD19^-$ $CD1c^+$
$CD304^+$ pDC	CD304 PE (from MACS), CD14 PE Cy7, CD19 PE-Cy7, Dapi	$Dapi^-$ $CD14^-$ $CD19^-$ $CD304^+$
$CD56^{bright}$ or $CD56^{dim}$ NK cells	CD3 PE, CD56 BV421, PI	bright: PI^- $CD3^-$ $CD56^{bright}$ dim: PI^- $CD3^-$ $CD56^{bright}$
naïve or memory $CD4^+$ T cells	CD4 ECD, CD45RO PE, CD45RA A488, CD62L APC-eFluor 780, Dapi	naïve: $Dapi^-$ $CD4^+$ $CD45RA^+$ $CD45RO^-$ $CD62L^+$ memory: $Dapi^-$ $CD4^+$ $CD45RO^+$ $CD45RA^-$
naïve $CD3^+$ T cells	CD3 PE, CCR7 A647, CD45RA A488, Dapi	$Dapi^-$ $CD3^+$ $CCR7^+$ $CD45RA^+$

In this work, different antibody panels for cell sorting by FACSAria II were used (Table 9). mDC were sorted for Dapi$^-$ CD14$^-$ CD19$^-$ CD1c$^+$ cells. pDC were sorted for Dapi$^-$ CD14$^-$ CD19$^-$ CD304$^+$ cells. NK cells were sorted for PI$^-$ CD3$^-$ CD56^{bright} or PI$^-$ CD3$^-$ CD56^{dim} cells. Naïve or memory CD4$^+$ T cells were sorted as Dapi$^-$ CD4$^+$ CD45RA$^+$ CD45RO$^-$ CD62L$^+$ or Dapi$^-$ CD4$^+$ CD45RO$^+$ CD45RA$^-$ cells respectively. Naïve complete T cells were sorted as Dapi$^-$ CD3$^+$ CCR7$^+$ CD45RA$^+$ cells. All sorted subsets displayed purity above 98%.

3.2.4.5. Flow cytometric analysis

In this work, data were acquired with two different flow cytometers from BD Biosciences: the LSR II and FACSCalibur. The LSR II was equipped with a blue laser (488nm), a red laser (633nm) and a violet laser (405nm). The FACSCalibur had two lasers: a blue one (488nm) and a red one (635nm). Data were analysed using the FlowJo software. Data were depicted in one-dimensional histograms or two-dimensional dot plots. When MSC were present in the culture, they were excluded from analysis by gating on CD45$^+$ cells.

3.2.5. Immune cell culture

Immune cell populations were cultured in RPMI-1640 medium supplemented with 10% AB serum, 100U/ml penicillin, 0.1mg/ml streptomycin, 2mM L-Glutamine and 20mM Hepes in tissue culture treated plates. Up to 1x10^5 cells were cultured in 200µl medium in 96 well plates. When MSC were present in the culture, flat bottom plates were used. Otherwise, immune cells were cultured in round bottom plates. Up to 1x10^6 cells were cultured in 1ml medium in 12 well plates. For some experiments, HTS Transwell plates (0.4µm pore size polycarbonate membranes or 1µm pore size polyester membrane) were used. In this case, mDC were seeded in the upper chamber and MSC or NK cells in the lower compartment.

3.2.6. Differentiation and maturation of moDC

1x10^6 monocytes were differentiated in the presence or absence of 1x10^5 BM-MSC (moDC to MSC ratio 10:1) in 1ml complete RPMI-1640 medium supplemented with 50ng/ml GM-CSF and 25ng/ml IL-4 in 12well plates. On day three, 50% of the medium

was replaced. After five days, moDC were matured by 100ng/ml LPS for another 24hrs. When BM-MSC were present only during the maturation process, $1x10^5$ cells were added together with the LPS on day five. LPS are components of the outer membrane of gram-negative bacteria and act as an endotoxin by binding to the CD14/TLR4/MD2 receptor complex, which is especially expressed by monocytes, DC and macrophages. TLR4 signalling is used to induce efficient DC maturation *in vitro*.

Expression of the surface markers CD14, CD40, CD80, CD83, CD86, HLA-DR and CCR7 after LPS maturation was analysed by flow cytometry at the LSR II (see sections 3.2.4.1 and 3.2.4.5). Transwell migration assay was performed towards CCL21 (see paragraph 3.2.9) and supernatants were analysed by cytometric bead array (CBA) for cytokine production (see section 3.2.10).

3.2.7. Maturation of pDC

$1x10^5$ pDC were co-cultured in the presence or absence of $2x10^4$ BM-MSC (pDC to BM-MSC ratio 5:1) in 200µl complete RPMI-1640 in 96 well flat bottom plates for 18hrs. Maturation was either induced by 5µg/ml CpG-B and 5µg/ml Resiquimod (R848) (for expression of maturation markers and migration assay) or by 10ng/ml IL-3 and 5µg/ml CpG-A (for cytokine analysis). The different stimuli were used due to different outcomes on pDC maturation [172]. CpG-A stimulation has been shown to induce high IFNα secretion, but low expression of maturation markers. On the contrary, CpG-B leads to high expression of maturation markers, but low production of IFNα. Both CpG molecules trigger TLR9 signalling in pDC.

Expression of surface molecules CD40, CD80, CD83, CD86, HLA-DR and CCR7 by mature pDC was analysed at the LSR II (see section 3.2.4.1 and 3.2.4.5). In order to study CCR7-dependet migration, a transwell migration assay was performed (see paragraph 3.2.9), while supernatants were analysed by multiplex analysis to measure secretion of cytokines, chemokines and growth factors (see section 3.2.11).

3.2.8. Maturation of mDC

$1x10^5$ mDC were co-cultured in the presence or absence of $2x10^4$ BM-MSC or PLX-PAD (mDC to MSC ratio 5:1) in 200µl complete RPMI-1640 in 96 well flat bottom plates for

18hrs. Maturation was either induced by a combination of 100ng/ml LPS and 10µg/ml R848 or by 100pg/ml LPS alone. LPS is used to induce TLR4 signalling, whereas R848 stimulates TLR7 and TLR8 signalling. It is known that mDC need high dose stimulation by using the combination of LPS and R848 to efficiently induce cytokine secretion *in vitro* [173]. This high dose maturation was used for all mDC experiments, except for the analysis of CCR7 expression and CCR7-dependent migration.

For some experiments, mDC and MSC were co-cultured in 96 well HTS Transwell plates to exclude MSC contaminations when mDC were used in a second step to activate NK or T cells. In this case, mDC were seeded in the upper chamber and MSC in the lower compartment. In some experiments, the following concentrations of blocking antibodies were added during mDC maturation: 20µg/ml anti-IL-10, 20µg/ml anti-IL-10 Receptor (IL-10R), 10µg/ml anti-interleukin-1 receptor antagonist (IL-1Ra) or 20µg/ml anti-IL-6 respectively.

Expression of surface markers CD40, CD80, CD83, CD86, HLA-DR and CCR7 by mature mDC was investigated at the LSR II (see sections 3.2.4.1 and 3.2.4.5). Transwell migration assay was performed to analyse CCR7-dependent migration (see paragraph 3.2.9) and supernatants were analysed by multiplex analysis to measure levels of cytokines, chemokines and growth factors (see section 3.2.11).

3.2.9. Migration assay

CCR7 is a chemokine receptor mediating migration of naïve T cells, DC and $CD56^{bright}$ NK cells into draining lymph nodes. The main ligands are the chemokines CCL19 and CCL21. Migration of mature moDC, pDC or mDC towards CCL21 was performed using 96 well HTS Transwell plates with 5µm pore size polycarbonate membranes as described before with minor changes [174]. $1x10^5$ DC were loaded in 70µl into the transwell insert and 200µl of complete RPMI-1640 medium supplemented with 125ng/ml CCL21 were added to the lower chamber. To determine basal migration, medium without chemokine was applied to the lower compartment. Plates were incubated for 90min at 37°C. In order to detach migrated cells from the membrane, EDTA was added into the lower compartment at a final concentration of 2mM for another 30min. Migrated cells from the lower chamber were collected, stained for CD45 and resuspended in exactly 200µl

PBS. CountBright Absolute Counting Beads and Dapi were added in order to determine the number of living migrated cells at the LSR II. $1x10^4$ beads were acquired in order to calculate the measured percentage of each sample. The final number of migrated DC was corrected for basal migration in the absence of CCL21.

3.2.10. Analysis of cytokine production using Cytometric Bead Array (CBA)

CBA is a bead-based immunoassay to measure simultaneously multiple cytokine concentrations by flow cytometry. Each type of capture bead is coated with antibodies specific for a single analyte. A combination of different beads and a mixture of detection antibodies, coupled to the reporter molecule PE, were mixed with the sample simultaneously. After 3hrs of incubation, the samples were washed and analysed. Due to their unique fluorescence intensity, it is possible to gate on the individual bead populations and calculate the mean fluorescence intensity (MFI) for the reporter molecule. By comparing the MFI values to a standard curve, the sample concentrations can be determined. Here, analysis of cell culture supernatants was performed using Human Inflammatory Cytokine Kit for moDC/BM-MSC co-cultures and Human Th1/Th2/Th17 Cytokine Kit for mDC/T cell co-cultures according to the manufacturer's instructions. Data were acquired on a FACSCalibur flow cytometer and analysed using FCAP Array v3 software.

3.2.11. Analysis of cytokine production using multiplex assay

Mutiplex assays allow the detection of more than 100 analytes of interest in one sample, requiring a very small sample volume. Microspheres or beads are internally stained with fluorescent dyes to produce a specific spectral address and are coated with antibodies to capture analytes of interest, for instance cytokines. These coupled beads react with the cytokine-containing samples. Biotinylated detection antibodies, which recognize the analytes of interest, create an antibody-antigen sandwich and are finally labelled by PE-conjugated streptavidin, which is used as reporter fluorescence. Using a flow cytometry based detection instrument, the beads are identified by one laser, while PE emission is detected by a second laser. Since the PE signal is directly proportional to the amount of

analyte bound, a standard curve can be used to determine the analyte concentrations of the samples.

Here, mDC/BM-MSC and pDC/BM-MSC co-culture supernatants were analysed using Bio-Plex Pro Human Cytokine Grp I Panel 27-Plex or a customized Cytokine Grp II Panel according to the manufacturer's instructions. All incubation steps were performed on an orbital shaker at 950rpm. After each step, beads were washed three times on a magnetic washer. Firstly, beads and samples were incubated at room temperature for 30min. Subsequently, detection antibodies were added for another 30min. Finally, PE-conjugated streptavidin was added for 10min. Acquisition and data analysis was done using the Bio-Plex Manager software.

3.2.12. Analysis of cytokine levels by enzyme-linked immunosorbent assay (ELISA)

The enzyme-linked immunosorbent assay (ELISA) is a biochemical assay to detect analytes, for instance cytokines, in liquids. Here, a sandwich ELISA was performed in the 96 well format according to the manufacturer's instructions. Between all incubation steps, the wells were washed five times with wash buffer. First, the plate was coated with the capture antibody in coating buffer overnight at 4°C. The wells were blocked with 1x assay diluent for one hour at room temperature. Serial dilutions of the standard were prepared according to the Certificate of Analysis to obtain a standard curve. Standards as well as samples were added to the appropriate wells and the plate was incubated overnight at 4°C. The diluted detection antibody was added at room temperature for one hour. Avidin-horseradish peroxidase was added to each well and the plate was incubated at room temperature for another 30min. Finally, substrate solution was added to each well and incubated at room temperature for 15min before adding stop solution to each well. The plate was read at 450nm and the values of 570nm were subtracted from those of 450nm.

3.2.13. T cell stimulation

3.2.13.1. $CD4^+$ T cell activation by allogeneic moDC

moDC that have been differentiated in the presence or absence of BM-MSC were used in a second step to activate sorted allogeneic naïve or memory $CD4^+$ T cells (Figure 9). $1x10^5$ T cells were co-cultured with $1x10^4$ moDC (T cell to moDC ratio 10:1) in 200µl complete RPMI-1640 in 96 well round bottom plates for different lengths of time, depending on the read out.

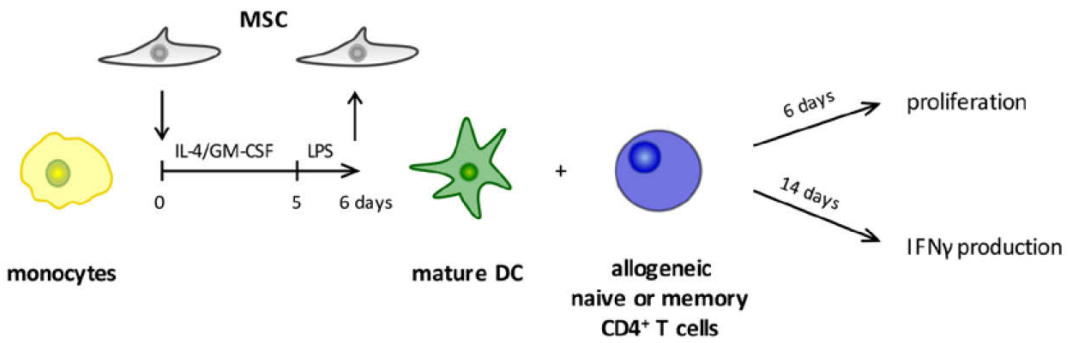

Figure 9: Activation of $CD4^+$ T cells by allogeneic moDC. moDC were generated in the presence or absence of MSC and used in a second step for T cell stimulation. T cells and mDC were co-cultured in a ratio of 10:1 for different time periods before analysis of proliferation or IFNγ production.

3.2.13.2. Analysis of $CD4^+$ T cell proliferation

For analysis of proliferation, allogeneic naïve or memory $CD4^+$ T cells were labelled with 5µM CFSE (see paragraph 3.2.4.3) before co-culture with mature moDC and cells were harvested after six days to analyse proliferation at the LSR II. The percentage of proliferated T cells was determined by gating on $Dapi^-$ $CD45^+$ $CD3^+$ $CD4^+$ $CFSE^-$ cells.

3.2.13.3. Analysis of IFNγ production by $CD4^+$ T cells

To measure cytokine production, sorted memory or naïve T cells were expanded in the presence of allogeneic moDC for 13 days in 96 well round bottom plates. After seven days, T cells were counted and splitted. $1x10^5$ cells per well were kept in culture in 200µl complete RPMI-1640 supplemented by 5ng/ml IL-7/IL-15. At day 13, $4x10^5$ expanded T cells were restimulated in the presence of 1µg/ml soluble anti-CD28 for 16hrs with

$4x10^5$ frozen monocytes (T cell to monocyte ratio 1:1). Monocytes derived from the same donor used for moDC generation. 10µg/ml Brefeldin A was added after 2hrs of restimulation to assess IFNγ production by intracellular staining (see section 3.2.4.2).

3.2.13.4. $CD3^+$ T cell stimulation by allogeneic mDC

mDC, which have been matured in the presence or absence of BM-MSC or PLX-PAD were used for stimulation of allogeneic naïve $CD3^+$ T cells (Figure 10). $1x10^5$ T cells were co-cultured with $2x10^4$ mature mDC (T cell to mDC ratio 5:1) in 200µl medium in 96 well round bottom plates for five days. Cytokine levels were analysed in cell culture supernatants by CBA (Human Th1/Th2/Th17 Cytokine Kit, see paragraph 3.2.10).

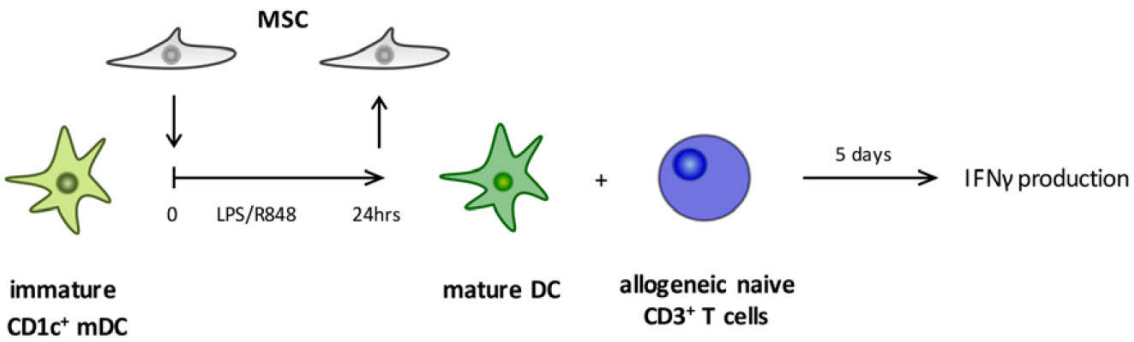

Figure 10: Stimulation of complete T cells by allogeneic mature mDC. mDC that have been activated in the presence or absence of MSC were used in a second step for T cell stimulation. T cells and mDC were co-cultured in a ratio of 5:1 for five days before analysis of IFNγ levels in the supernatant.

3.2.14. NK cell activation

3.2.14.1. NK cell stimulation by autologous mDC

mDC that have been matured in the presence or absence of BM-MSC or PLX-PAD were used in a second step for NK cell activation (Figure 11). NK cells and mDC were co-cultured in a ratio of 2:1 in 200µl complete RPMI-1640 medium for different time periods depending on the effector function analysed. For some experiments, the following concentrations of blocking antibodies were added during NK cell stimulation by mDC: 20µg/ml anti-IL-10, 20µg/ml anti-IL-10R, 10µg/ml anti-IL-1Ra or 20µg/ml anti-IL-6 respectively.

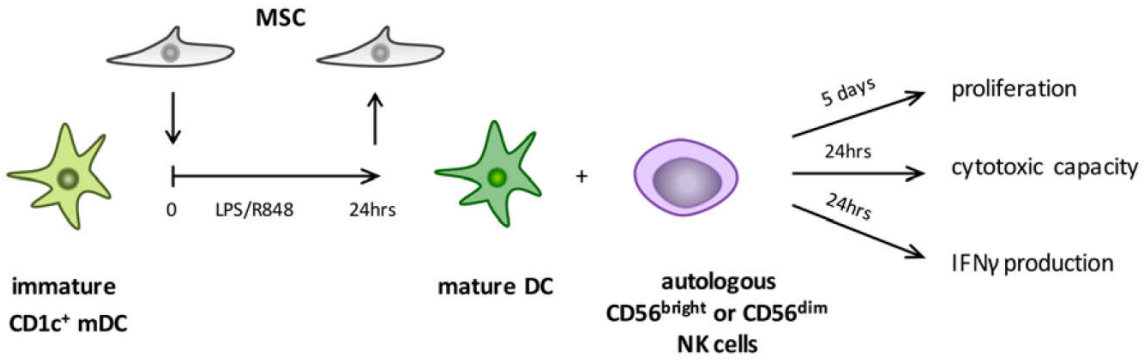

Figure 11: NK cell stimulation by autologous activated mDC. mDC that have been matured in the presence or absence of MSC were used in a second step for NK cell stimulation. NK cells and mDC were co-cultured in a ratio of 2:1 for different time periods before analysis of proliferation, cytotoxicity of mDC-primed NK cells towards MHC-I negative target cells K562 and IFNγ production.

3.2.14.2. Analysis of IFNγ production by NK cells

$5x10^4$ $CD56^{dim}$ or $CD56^{bright}$ NK cells were co-cultured with $2,5x10^4$ mature mDC in 200µl medium in 96 well round bottom plates for 24hrs. 10µg/ml Brefeldin A was added for the last 8hrs before harvesting for intracellular staining of IFNγ (see paragraph 3.2.4.2).

3.2.14.3. Investigation of cytotoxic potential of NK cells

The MHC class I negative tumour cell line K562 is frequently used as target cells to analyse specific lysis by NK cells *in vitro*. Non-adherent K562 cells were cultured for at least two days in 10ml complete RPMI-1640 in tissue culture dishes before being used for cytotoxicity assays. NK cells and K562 cells were seeded in an effector to target ratio of 5:1.

The cytotoxic potential of NK cells can be enhanced by preactivation with mDC. Therefore, $1x10^5$ $CD56^{dim}$ NK cells were co-cultured with $5x10^4$ mature mDC in HTS Transwell 96 well plates for 18hrs (NK to mDC ratio 2:1). mDC were seeded in the transwell insert and NK cells in the lower chamber. When higher NK cell numbers were required, several wells were seeded. Subsequently, NK cells were counted and a CD107a Mobilization Assay or a flow cytometric assay for NK cell killing was performed as described before [175,176].

To measure CD107a expression by NK cells, $1x10^5$ NK cells were co-cultured with $2x10^4$ K562 cells in 120µl complete RPMI-1640 medium in a 96 well round bottom plate in the

presence of anti-CD107a Fitc, 1µl/ml GolgiStop (containing Monensin) and 10µg/ml Brefeldin A at 37°C for 6hrs. Cells were harvested, stained for CD56 and analysed at the LSR II after addition of PI.

Moreover, specific killing of K562 cells was quantified. K562 target cells were stained with 5µM CFSE before co-culture with NK cells to facilitate gating during FACS analysis. $5x10^5$ NK cells were incubated with $1x10^5$ K562 cells in 24 well plates with transwell inserts at 37°C for 6hrs. After washing the cells, PI was added and dead K562 target cells (Td) were identified as $CFSE^+$ PI^+ at the LSR II. Specific lysis was calculated as $Td_{(cultured\ with\ effector\ cells)}$ -$Td_{(cultured\ without\ effector\ cells)}$.

3.2.14.4. Analysis of NK cell proliferation

Sorted $CD56^{dim}$ or $CD56^{bright}$ NK cells were labelled with 1µM CFSE and $1x10^5$ labelled cells were co-cultured with $5x10^4$ mature mDC in 200µl medium in 96 well round bottom plates for five days (NK to mDC ratio 2:1). After addition of PI, proliferation was measured at the LSR II by gating on $CD56^+$ $CFSE^-$ PI^- cells.

3.2.15. Detection of IFNγ-producing T cells by using Enzym-linked immunosorbent spot (Elispot) assay

The Elispot assay is a sensitive method to quantify the number of cytokine producing cells, for instance IFNγ-producing memory T cells. Cell suspensions (e.g. PBMC) are cultured in the presence of an antigen of interest on a surface that is coated with a primary capture antibody (Figure 12). Cells that are specific for the tested antigen produce cytokines. These are bound by the immobilized antibodies in the immediate vicinity of the secreting cells. The cells are washed away and a secondary detection antibody, which is coupled to a chromogen, is used to detect the bound cytokine. It is visualized by a colour reaction, leading to spots that can be quantified by an automated Elispot reader. Each spot represents an individual cytokine-secreting cell (Figure 12). Here, the T cell IFNγ Elispot was used to analyse if CLI patients that have been treated with PLX-PAD cells develop a memory T cell response specific for the PLX-PAD donor. IFNγ is the signature cytokine of the Th1 lineage.

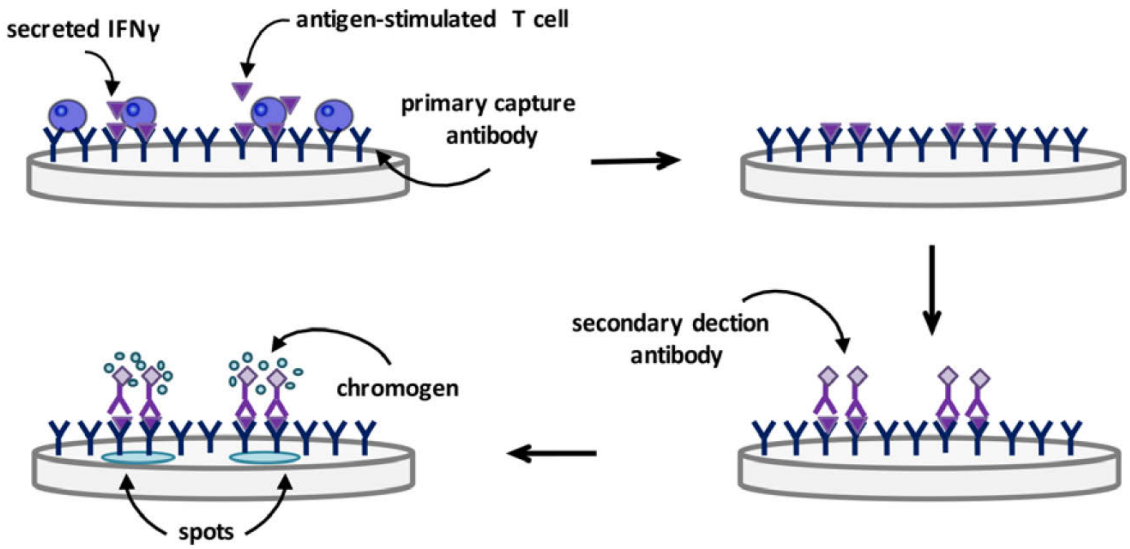

Figure 12: Detection of IFNγ-producing T cells by Elispot assay. Antigen stimulated memory T cells produce IFNγ, which is captured by the membrane-bound primary antibodies and detected by the secondary antibodies. The chromogen is used for visualization and spots can be quantified using an automated Elispot reader.

The T cell IFNγ Elispot was carried out by Maik Stein from the Division of Nephrology at the Berlin-Brandenburg Center for Regenerative Therapies (BCRT) and data were kindly provided for analysis. PBMC from CLI patients were stored at liquid nitrogen before being used in the Elispot assay. $3x10^5$ thawed PBMC per well were stimulated in triplicates in 200µl complete RPMI-1640 medium in Elispot plates by $6x10^4$ PLX-PAD or $6x10^4$ third-party allogeneic PBMC per well. Non-stimulated PBMC were used as negative control (background). To ensure the functionality of frozen/thawed T cells, SEB-stimulated samples were run as positive control. After incubation at 37°C for 24hrs, the cells were removed and wells were washed six times before addition of 100µl secondary detection antibody. Plates were incubated at room temperature for 2hrs and washed again six times. 100µl substrate solution per well were added and plates were incubated at room temperature for 5min until spots became clearly visible but were still separated from each other. The reaction was stopped by washing three times with tap water. Plates were read at the ImmunoSpot reader. As described before, a response of more than 25 IFNγ spots/$3x10^5$ PBMC was considered as clinically relevant [177]. Data were excluded from analysis if no sample pairs before/ after (day three/ week one/ week four) PLX-PAD treatment were available or if they did not fit the quality control criteria. These were enough living PBMC after thawing and at least 100 IFNγ spots/$3x10^5$ PBMC in SEB-stimulated positive controls.

3.2.16. Detection of MSC in human tissue sections by chipcytometry

Chipcytometry is a technique that combines flow cytometry and microscopy and has been developed by the biotech company *Zellkraftwerk* [178]. Commonly used flow cytometry is limited to approximately 12-15 biomarkers that can be analysed at the same time, while chipcytometry enables investigation of up to 30 parameters at the same time on one slide. Furthermore, biomarkers are fixed at the time of preparation and can be reanalysed for about twelve months allowing application of new antibodies months later. Chipcytometry allows analysis of cell suspensions (using ZellSafe_C chips) or tissue cryosections (using ZellSafe_T chips).

3.2.16.1. Loading of cultured human BM-MSC onto Zellsafe_C chips for chipcytometry

In order to establish the antibody staining to detect MSC in human tissue cryosections, cultured BM-MSC were loaded onto Zellsafe_C chips. In order to simulate the *in vivo* situation in tissue samples and test the detection limit of this method, PBMC were spiked with BM-MSC to get an approximate dilution of 50,000 PBMC to one BM-MSC. The company Zellkraftwerk performed the antibody staining (Table 10) and sample analysis.

Table 10: Antibodies used for detection of human MSC by chip cytometry.

Antibody (Clone)	Company	MSC expected to be
CD73 (AD2)	BioLegend	positive
CD90 (SE10)	BioLegend	positive
CD105 (43A3)	BioLegend	positive
CD14 (RMO52)	Beckman Coulter	negative
CD19 (HIB19)	eBioscience	negative
CD34 (561)	BioLegend	negative
CD45 (HI30)	BioLegend	negative
HLA-DR (G46-6)	BD Biosciences	negative

Freshly cultured BM-MSC (passage three) were trypsinized prior to application onto Zellsafe_C chips and treated according to the standard operating procedure (SOP) “Preparation and fixation of short term cultured cells on ZellSafe_C Chips” (version 1.01). For some chips, PBMC were prepared according to the standard procedure (see

paragraph 3.2.2) and spiked with BM-MSC to get an approximate dilution of 50,000:1. The cell pellet was resuspended in 500µl Zellkraftwerk relaxation buffer in an Eppendorf tube, left untouched for 15min at room temperature. Cells were centrifuged and the cell pellet was resuspended in 40µl Zellkraftwerk wash buffer. The pipetting adapter was plugged into the inlet of the chip and the chip was rinsed three times with 200µl Zellkraftwerk wash buffer. The cell solution was applied onto the chip and incubated for 5min at 4°C. The chip was washed carefully twice by applying 200µl Zellkraftwerk wash buffer drop wise onto the pipetting adapter to remove unbound cells and debris. Cells were fixed by carefully adding 200µl Zellkraftwerk fixation buffer twice and incubated for 15min at 4°C. The chip was rinsed four times with 200µl Zellkraftwerk storage buffer before sealing it. Chips were stained for CD73, CD90 and CD105 (positive markers) as well as CD14, CD19, CD34, CD45 and HLA-DR (negative markers) one after the other by Zellkraftwerk (Figure 13 and Figure 38).

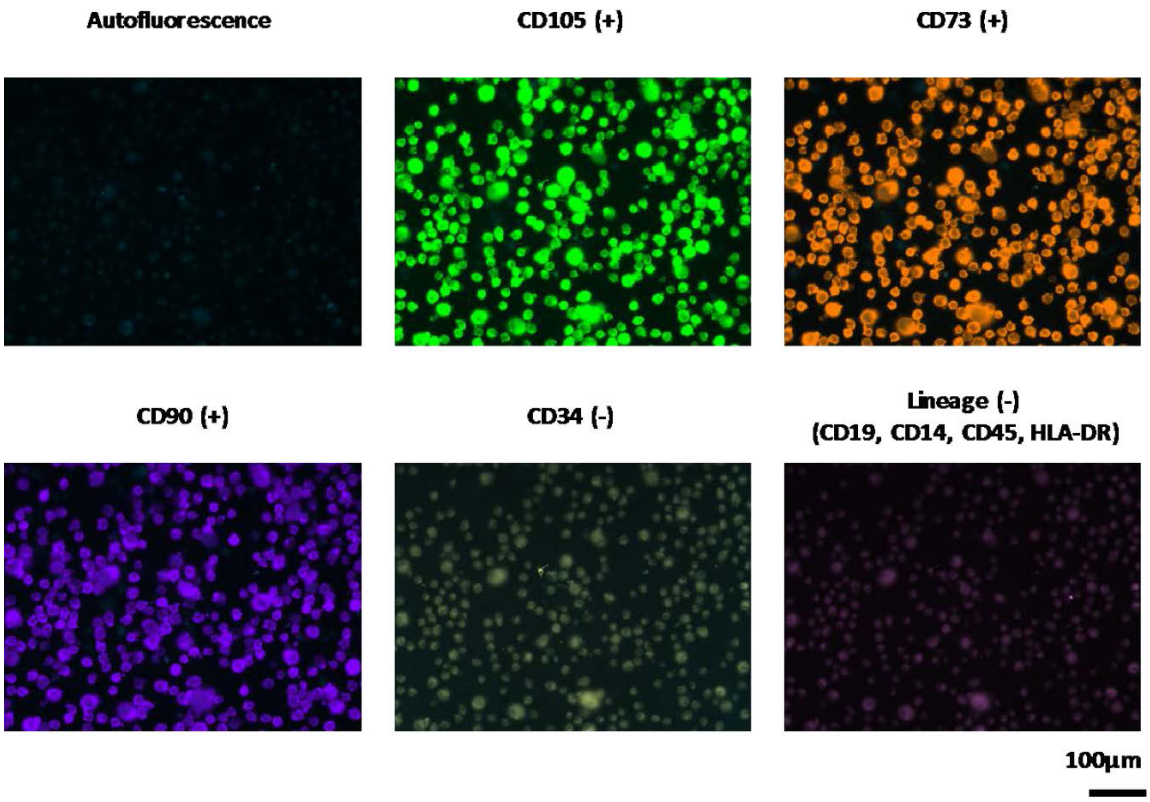

Figure 13: Establishment of antibody stainings on *in vitro* cultured BM-MSC. Cultured human BM-MSC were loaded onto Zellsafe_C chips and stained for CD73, CD90 and CD105 (positive markers) as well as CD14, CD19, CD34, CD45 and HLA-DR (negative markers) one after the other.

3.2.16.2. Preparation of human placenta tissue sections for chip cytometry

Frozen tissue sections from human placentas were generated in cooperation with Markus Scharm from the lab for Experimentelle Gynäkologie und Geburtshilfe Medizinische Fakultät at Otto-von-Guericke Universität Magdeburg. Placentas either derived from abortions (11^{th} to 12^{th} gestation week, hereafter termed early pregnancy) or full term pregnancy (hereinafter called late pregnancy) and were embedded in optimal cutting temperature (OCT) compound. The sections were treated according to the SOP "Preparation and fixation of tissue cryosections on ZellSafe_T chips" (version 1.1). 5µm cryosections were prepared and transferred on 24x46mm cover slips. Sections were dried at 37°C for 15min. In order to remove OCT compound, samples were washed 15 to 20min in PBS on a shaker before changing the buffer against Zellkraftwerk wash buffer for another 5min. The ZellSafe_T chip was cleaned with 70% ethanol and a lint wipe. The protection film was removed and the dry cover slip was placed onto the chip, the tissue section facing the channel. A pipetting adapter was placed in one of the inlets and 1ml Zellkraftwerk fixation buffer was applied very slowly to the channel. The chip was incubated for 15min at 4°C before rinsing the chip twice with 1ml Zellkraftwerk wash buffer. Finally, 1ml Zellkraftwerk storage buffer was applied twice to the chip before sealing the chip. Samples were stained and analysed by the company Zellkraftwerk.

3.2.17. Statistical analysis

Statistical analysis was performed using GraphPad Prism. Detailed information on the sample size is provided in each figure legend. All values in the figures are presented as mean ± standard error mean (SEM) unless otherwise noted in the figure legends. Statistical significance was calculated using Wilcoxon signed rank test. The levels of significance were set as $P<0.05$ (*), $P<0.005$ (**) and $P<0.0005$ (***).

4. RESULTS

4.1. Influence of BM-MSC on *in vitro* differentiation and maturation of human moDC

Immature DC patrol the body until they encounter their antigen and start the maturation process, characterized by three main properties: upregulation of costimulatory molecules and molecules required for efficient antigen presentation, acquisition of the chemokine receptor CCR7 in order to migrate towards draining lymph nodes and production of pro-inflammatory cytokines and chemokines.

To date, almost all published *in vitro* studies on the influence of MSC on human DC have been performed with DC that were generated from monocytes or $CD34^+$ precursors (see section 1.3.2). It has clearly been shown that MSC inhibit DC differentiation of moDC *in vitro*, whereas there are contradictory reports regarding their effects on DC maturation [137,139]. Here, we investigated how BM-MSC affect expression of maturation markers, CCR7-dependent migration and cytokine production of *in vitro* generated moDC. Finally, we analysed if BM-MSC also affect the ability of moDC to activate T cells.

4.1.1. BM-MSC inhibit differentiation but not maturation of *in vitro* generated moDC

We analysed the influence of BM-MSC on the expression of the maturation markers CD40, CD80, CD83, CD86 and HLA-DR when they were either present during the whole process of *in vitro* differentiation and maturation or only during the maturation of human $CD14^+$ monocytes into $CD14^-$ moDC. In accordance to other publications [135,179], we observed a significantly higher remaining percentage of CD14 expressing cells when BM-MSC have been present during differentiation (Figure 14). This shows that monocytes mainly remained undifferentiated or insufficiently differentiated when co-cultured with BM-MSC. As a consequence of the incomplete differentiation, moDC also acquired less expression of CD40, CD83 and CD86 while CD80 as well as HLA-DR expression were not altered. In contrast, we could not detect an effect of BM-MSC on the expression of all analysed maturation markers when they have been present only during the maturation of already differentiated immature moDC (Figure 14).

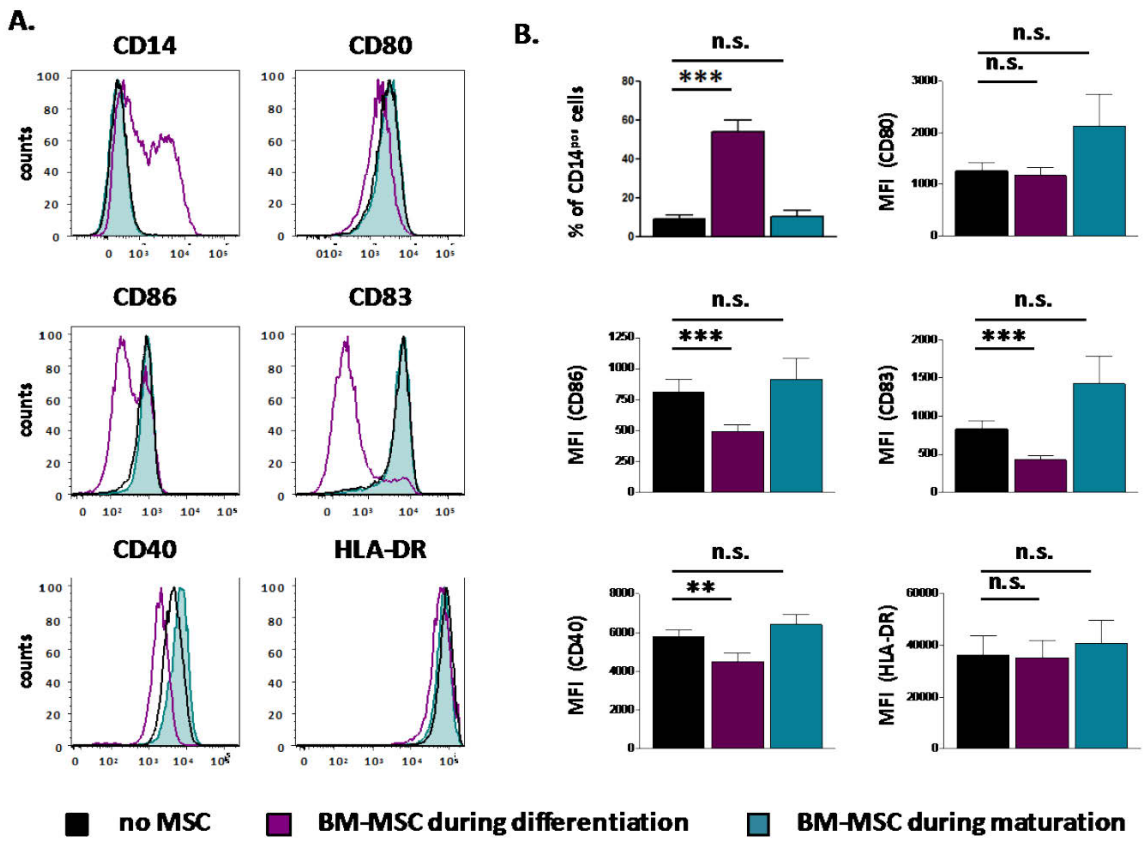

Figure 14: BM-MSC inhibit differentiation but not maturation of *in vitro* generated moDC. (A-B) Human moDC were generated from $CD14^+$ monocytes by GM-CSF/IL-4 for five days and subsequently matured by LPS for another 24hrs. Expression of CD14, CD86, CD80, CD83, CD40 and HLA-DR was analysed by flow cytometry. BM-MSC were either present during differentiation/maturation (violet, n=20) or only during maturation of moDC (blue, n=6). **(A)** Histograms for one representative experiment are shown. **(B)** Mean expression levels of the specified markers by moDC +/- SEM are shown. P<0.005 (**) and P<0.0005 (***).

4.1.2. BM-MSC only decrease CCR7-dependent migration of moDC when present during differentiation

Circulating immature DC are $CCR7^-$ and only upregulate its expression upon maturation stimuli. So far, there are no data available on the influence of MSC on CCR7-dependent migration of human DC. Therefore, we analysed if BM-MSC impair migration of moDC towards the receptor's ligand CCL21 when they were either present during differentiation or only during maturation. As shown in Figure 15A and Figure 15B, approximately 20% of moDC expressed CCR7 after LPS-induced maturation *in vitro*. When BM-MSC were present during differentiation, which caused a strong inhibition of DC generation, the percentage of $CCR7^{high}$ moDC as well as the MFI of the $CCR7^{high}$ cells were decreased (Figure 15A and Figure 15B). In contrast, when BM-MSC were present only during the maturation process, we observed an enhanced CCR7 acquisition, which

was represented by the percentage of CCR7^{high} moDC as well as the MFI of the CCR7^{high} cells. These observations correlated with the number of migrated cells when performing a migration assay towards CCR7 ligand CCL21 (Figure 15C). The number of migrated moDC was strongly reduced when BM-MSC were present during differentiation while it was increased when BM-MSC were present only during the maturation process.

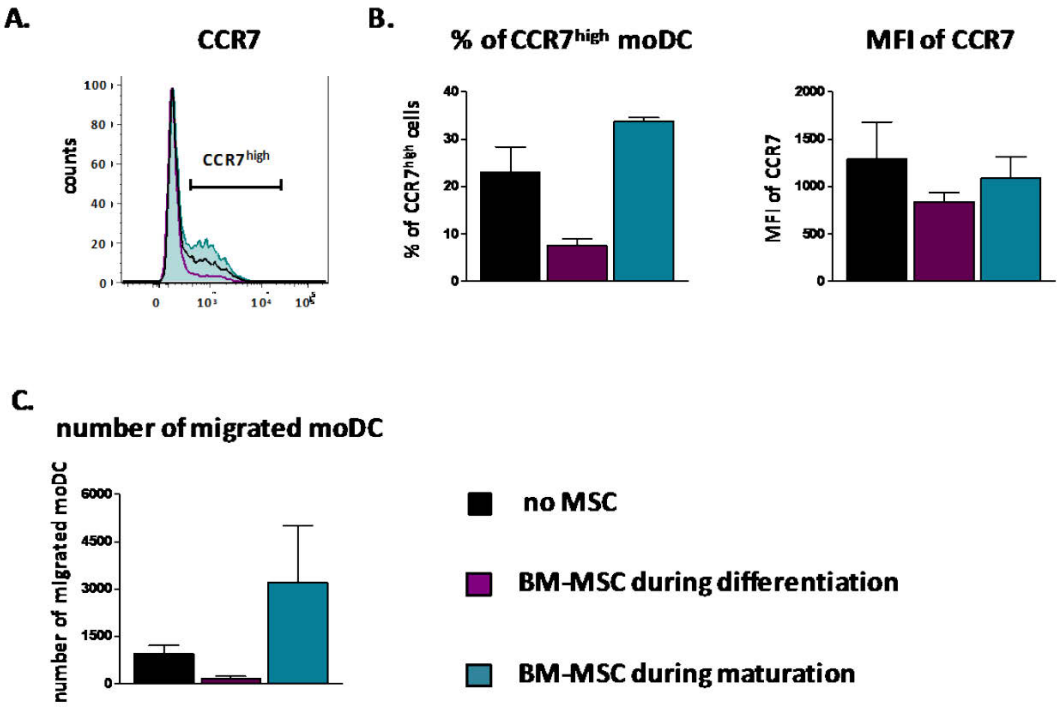

Figure 15: BM-MSC inhibit CCR7-dependent migration of moDC when present during differentiation. (A-C) CCR7 expression of LPS-matured moDC was analysed by flow cytometry. Transwell migration assay was performed towards CCL21. moDC were generated in the absence of BM-MSC (black) or BM-MSC were either present during differentiation/maturation (violet) or only during maturation of moDC (blue). **(A)** The histogram shows CCR7 expression for one representative donor out of three. **(B)** The percentage of CCR7^{high} moDC as well as the MFI of CCR7^{high} cells are shown as mean +/- SEM. **(C)** The mean number of migrated moDC is depicted +/- SEM (n=5).

4.1.3. moDC differentiated in the presence of BM-MSC secrete less pro-inflammatory cytokines

The production of pro-inflammatory cytokines by DC leads to the activation of effector cells like NK or T cells. In this way, the secretion profile of DC has an import regulatory impact on the course of an immune response. Using CBA, we measured the levels of different cytokines in the culture supernatant of moDC that have been generated in the presence or absence of BM-MSC. Similar to what has been shown by Spaggiari et al. for IL-12 production [137], we observed that moDC secreted lower amounts of IL-12p70,

IL-1β and TNFα upon LPS activation when BM-MSC were present during the differentiation process (Figure 16).

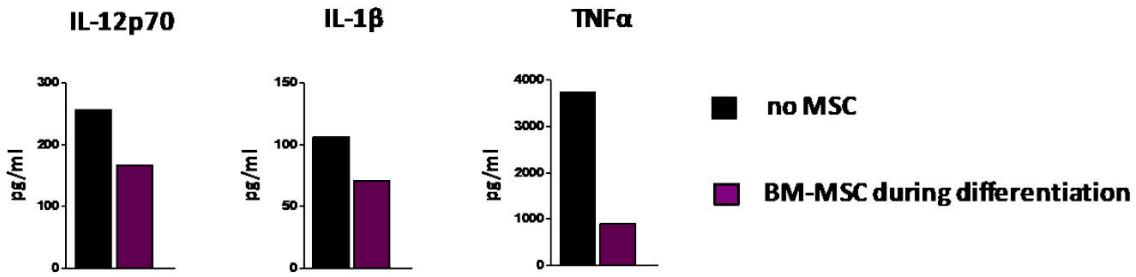

Figure 16: moDC differentiated in the presence of BM-MSC secrete less pro-inflammatory cytokines. moDC were generated in the presence (violet) or absence (black) of BM-MSC and matured by LPS. Levels of IL-12p70, IL-1β and TNFα in culture supernatants were determined by CBA (n=1).

4.1.4. moDC matured in the presence of BM-MSC display a reduced potential to induce T cell proliferation and Th1 priming

Since we observed a decreased activation of moDC when BM-MSC were present during the differentiation process, we investigated how BM-MSC affect moDC's ability to activate allogeneic naïve or memory $CD4^+$ T cells.

Here, we analysed the proliferative capacity of $CD4^+$ T cells, as measured by CFSE dilution after six days of co-culture (Figure 17A). Roughly 60% of naïve $CD4^+$ T cells proliferated when they were stimulated by allogeneic moDC. This percentage was significantly reduced when T cells were activated by allogeneic moDC that have been differentiated in the presence of BM-MSC. When memory $CD4^+$ T cells were stimulated by moDC, about 40% of T cells proliferated. This number was also significantly lower when BM-MSC were present during moDC generation.

Moreover, we analysed how BM-MSC influence the capacity of moDC to induce cytokine production in allogeneic naïve or memory $CD4^+$ T cells *in vitro*. As depicted in Figure 17B, moDC that were matured in the presence of BM-MSC were less efficient in inducing Th1 priming of naïve T cells as shown by a reduced percentage of $IFN\gamma^+$ T cells after 14 days of co-culture and subsequent restimulation for 16hrs before intracellular cytokine staining. In contrast, we detected the same percentage of IFNγ-producing memory T cells when stimulated by moDC or moDC that were differentiated in the presence of BM-MSC.

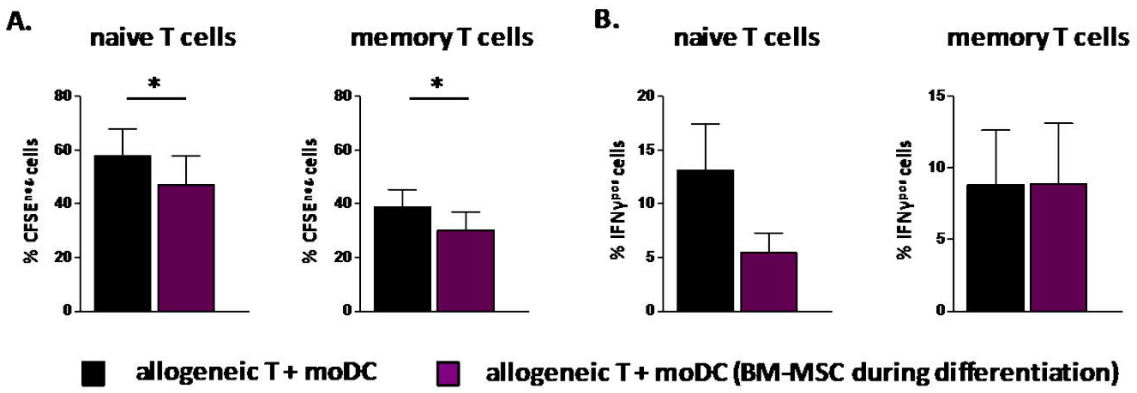

Figure 17: moDC that were matured in the presence of BM-MSC induce less proliferation and Th1 priming in allogeneic CD4$^+$ T cells. (A-B) LPS-matured moDC were generated in the presence (violet) or absence (black) of BM-MSC before being used for activation of allogeneic naïve or memory CD4$^+$ T cells. **(A)** The percentage of CFSE$^-$ T cells was determined by flow cytometry at day six and is depicted as mean +/- SEM (n=8). **(B)** T cells were co-cultured with the indicated allogeneic moDC for 14 days before being restimulated with monocytes from the moDC donor for 16hrs. Intracellular staining for IFNγ was analysed by flow cytometry. The percentage of IFNγ producing T cells is presented as mean +/- SEM (n=3). P<0.05 (*).

4.2. Effect of BM-MSC on *in vitro* maturation of human pDC

Since it has been shown *in vitro* and *in vivo* that moDC resemble rather inflammatory than conventional DC, they do not seem to be a good *in vitro* system to simulate the *in vivo* situation for MSC applications. Other *ex vivo* isolated DC subsets might be a more adequate model system to study MSC effects. One type of DC that can readily be isolated from human blood are CD304$^+$ (BDCA4) pDC (see paragraph 1.2.2.2), which produce high amounts of IFNα and are one of the key players for antiviral immune responses [77]. We analysed the impact of BM-MSC on the acquisition of maturation markers, CCR7-dependent migration and the secretion of cytokines, growth factors and chemokines.

4.2.1. BM-MSC have no clear effect on *in vitro* maturation of pDC

As shown in Figure 18A, freshly isolated pDC expressed HLA-DR, but lacked expression of CD80, CD86, CD83 and CD40. This represents an expression profile typical for immature DC. After activation by TLR ligands CpG-B/R848, which are commonly used to induce maturation marker expression by pDC in vitro [180], pDC displayed a high expression of CD40 and HLA-DR. However, compared to moDC and mDC (see paragraph 4.1.1, Figure 14 and section 4.3.1, Figure 22), pDC acquired rather low levels of CD80, CD83 and CD86.

When BM-MSC were present during the maturation process ([BM-MSC]pDC), pDC expressed comparable amounts of the maturation markers CD40, CD86 and HLA-DR, while the levels of CD80 and CD83 were slightly increased (Figure 18).

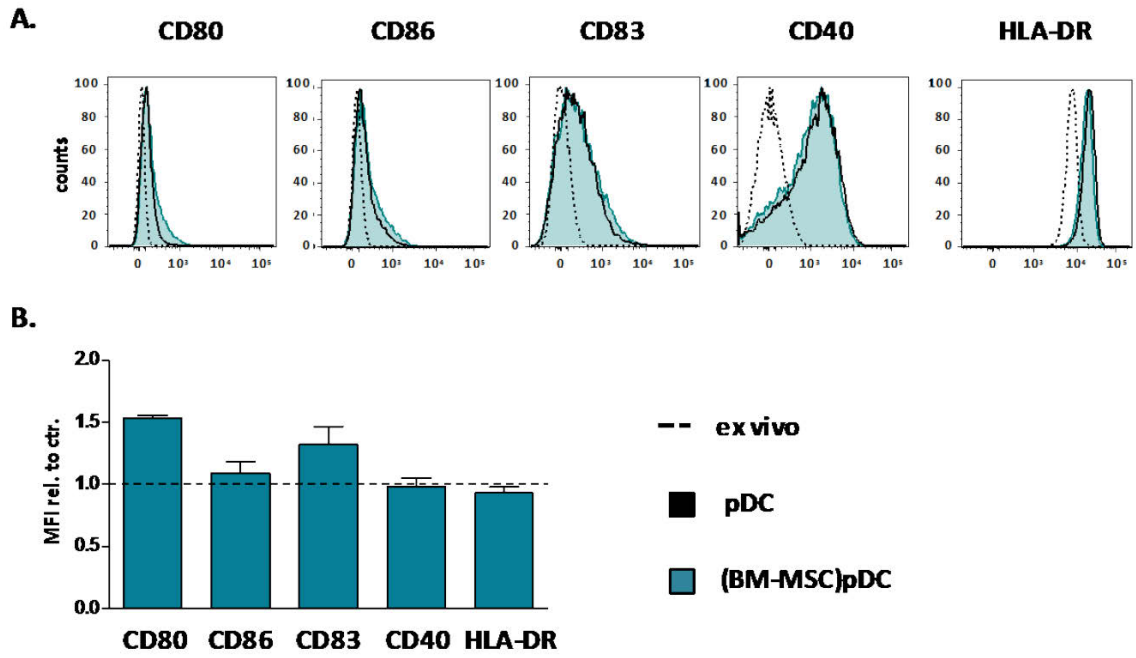

Figure 18: BM-MSC do not have a clear effect on maturation of *in vivo* differentiated pDC. (A-B) pDC were stimulated by CpG-B/R848 in the presence (blue) or absence (black) of BM-MSC for 18hrs. Acquisition of CD86, CD80, CD83, CD40 and HLA-DR by pDC was investigated by flow cytometry (n=3). **(A)** Data for one representative experiment are shown for *ex vivo* isolated pDC (dotted line) as well as for mature pDC and (BM-MSC)pDC. **(B)** Mean MFI values for the indicated cell surface markers of (BM-MSC)pDC relative to control (rel. to ctr.) pDC +/- SEM are depicted.

4.2.2. BM-MSC decrease CCR7-dependent migration of pDC

Freshly isolated pDC did not express CCR7, while almost 100% of pDC acquired CCR7 expression when matured by CpG-B/R848 (Figure 19A). This percentage was not altered when BM-MSC were present during the maturation process. The CCR7 level expressed per cell, as reflected by MFI of the $CCR7^{high}$ cells, was even slightly increased in (BM-MSC)pDC (Figure 19A and Figure 19B). Though, when performing a transwell migration assay towards CCR7 ligand CCL21, the number of migrated (BM-MSC)pDC was significantly lower compared to pDC that were matured in the absence of BM-MSC.

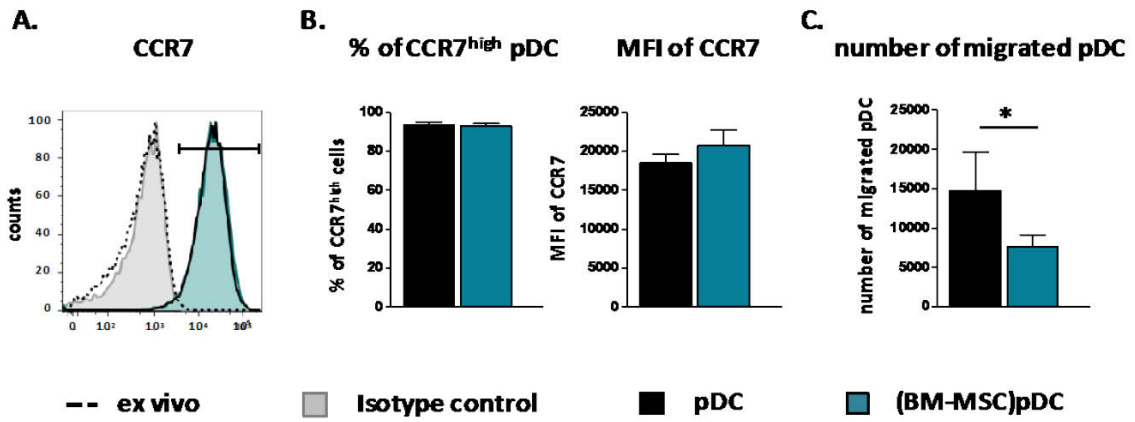

Figure 19: BM-MSC decrease CCR7-dependent migration of pDC. (A-C) pDC were matured by CpG-B/R848 for 18hrs. The CCR7 level was analysed by flow cytometry. Transwell migration assay was performed towards CCL21. **(A)** The histogram presents data for pDC (black), (BM-MSC)pDC (blue), *ex vivo* isolated pDC (dotted line) and the corresponding isotype control (light grey) for one representative experiment. **(B)** The percentage of $CCR7^{high}$ pDC and the MFI of $CCR7^{high}$ pDC are shown as mean +/- SEM (n=11). **(C)** The number of migrated pDC is depicted as mean +/- SEM (n=7). P<0.05 (*).

4.2.3. BM-MSC enhance production of IFNα and chemokines by pDC

It is known that pDC get activated by different regulatory pathways when stimulated by synthetic oligonucleotides CpG-A or CpG-B *in vitro* [77,180]. Cytokine production, and especially release of IFNα, needs stimulation by CpG-A. We matured pDC in the presence or absence of BM-MSC by IL-3/CpG-A and measured concentrations of cytokines, chemokines and growth factors in the supernatants by multiplex analysis or, in the case of IFNα, by ELISA (Table 11). Selected results are shown in Figure 20 and Figure 21.

As depicted in Figure 20A, pDC produced higher amounts of their signature cytokine IFNα in the presence of BM-MSC. Moreover, we measured significantly less TNFα and more IL-6 in the co-culture between pDC and BM-MSC (Figure 20B). We also detected increased levels of the chemokines IP-10, monocyte chemotactic protein 1 (MCP-1) and IL-8 (Figure 21A) in pDC/BM-MSC co-cultures compared to pDC alone. Additionally, we measured an increased VEGF secretion in pDC/BM-MSC co-cultures (Figure 21B). Yet, we already observed a high VEGF level when BM-MSC were cultured alone, indicating that this growth factor is rather produced by BM-MSC than by pDC. Some other measured factors also showed significant differences between pDC alone and pDC/BM-MSC co-cultures, but the overall concentration was at the lower detection limit. For this reason, they were not considered for interpretation (Table 11).

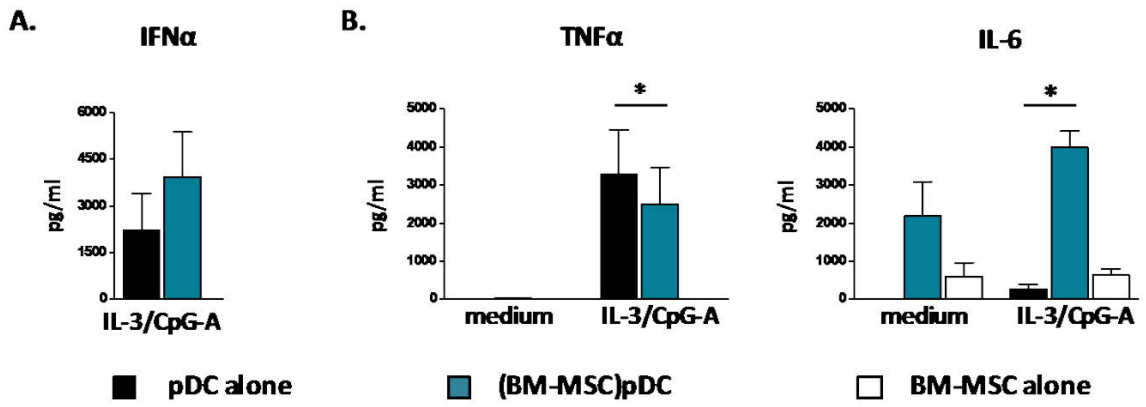

Figure 20: BM-MSC enhance production of IFNα and IL-6, but decrease secretion of TNFα by pDC. (A-B) pDC alone (black), (BM-MSC)pDC (blue) or BM-MSC alone (white) were cultured in medium with or without IL-3/CpG-A for 18hrs. Cytokine production in pDC/BM-MSC co-culture supernatants was determined by ELISA **(A**, n=5) or multiplex assay **(B**, n=13). Concentrations of the indicated soluble factors are presented as mean +/- SEM. P<0.05 (*).

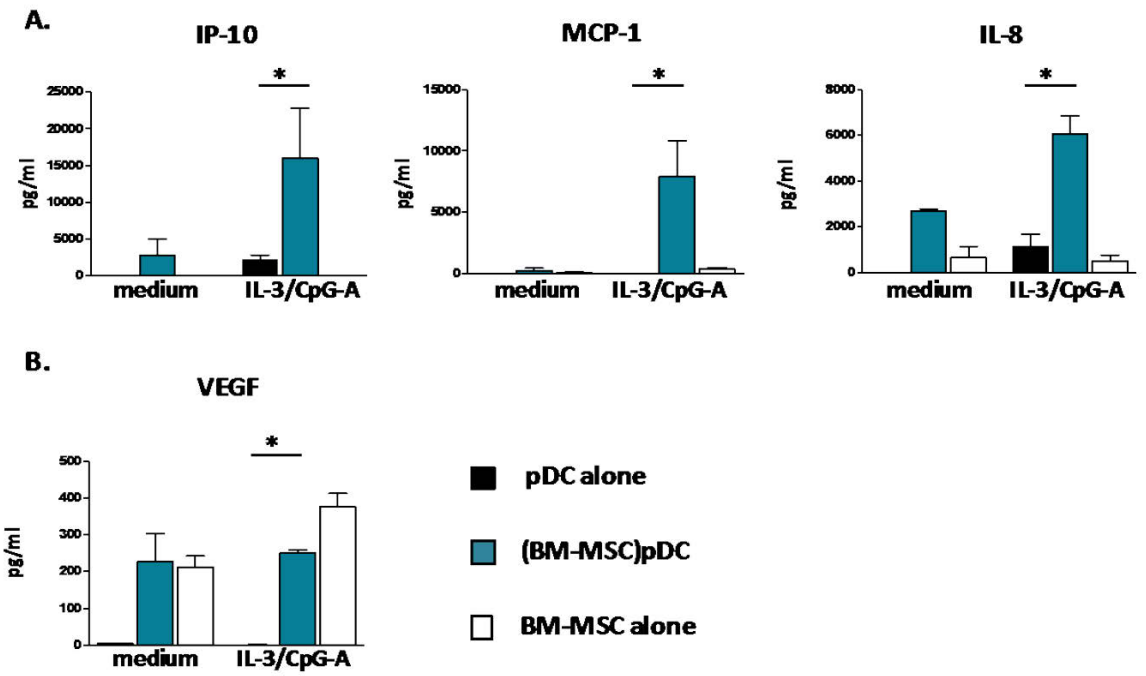

Figure 21: BM-MSC increase release of chemokines and growth factors by pDC. (A-B) pDC alone (black), (BM-MSC)pDC (blue) or BM-MSC alone (white) were cultured in medium with or without IL-3/CpG-A for 18hrs. Concentrations of chemokines and growth factors in pDC/BM-MSC co-culture supernatants were measured by performing a multiplex assay (n=13). Concentrations of the indicated factors are shown as mean +/- SEM. P<0.05 (*).

Table 11: Analysis of cytokines, chemokines and growth factors in pDC/BM-MSC co-culture supernatants. Cytokine levels in pDC/BM-MSC co-culture supernatants were determined by multiplex analysis (n=2-7). pDC alone, (BM-MSC)pDC or BM-MSC alone were cultured in medium with or without IL-3/CpG-A for 18hrs. Concentrations of the indicated soluble factors are shown as mean +/- SEM (pg/ml). Significance was tested between IL-3/CpG-A matured pDC and pDC/BM-MSC co-cultures. *Abbreviations: ctr., control; n.d., not detectable; n.s., not significant, P<0.05 (*).*

Analyte	pDC ctr. (pg/ml)	BM-MSC ctr. (pg/ml)	pDC/BM-MSC ctr. (pg/ml)	pDC IL-3/CpG-A (pg/ml)	BM-MSC IL-3/CpG-A (pg/ml)	pDC/BM-MSC IL-3/CpG-A (pg/ml)	Significance
Eotaxin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	not tested
IFNγ	n.d.	n.d.	13.5 ± 13.5	15.0 ± 8.8	n.d.	82.6 ± 38.8	*
bFGF	n.d.	8.7 ± 5.7	29.6 ± 1.3	7.6 ± 4.9	n.d.	23.3 ± 3.5	*
G-CSF	n.d.	2.4 ± 2.4	24 ± 1.9	7.4 ± 5.9	n.d.	14.3 ± 9.1	not tested
GM-CSF	12.0 ± 3.1	5.5 ± 3.7	15.1 ± 1.0	2 ± 1.5	2.8 ± 2.8	10.5 ± 2.8	not tested
IL-1β	n.d.	n.d.	n.d.	4.8 ± 1.1	n.d.	4.2 ± 0.9	not tested
IL-1ra	n.d.	n.d.	16.1 ± 16.1	42.3 ± 16.1	n.d.	90.0 ± 21.5	*
IL-6		603.2 ± 350.5	2,181 ± 899.5	261.2 ± 136.2	629.2 ± 167.8	3,976 ± 436.8	*
IL-8	3.3 ± 3.3	649.3 ± 486.7	2,669 ± 109.2	1131 ± 541.2	494.9 ± 276.0	6,071 ± 762.5	*
IL-10	2.2 ± 0.1	3.0 ± 1.9	7.4 ± 4.2	n.d.	5.2 ± 5.2	1.8 ± 1.8	not tested
IL-12p70	n.d.	15.3 ± 5.8	21 ± 6	1.6 ± 1.6	28.3 ± 12.0	10.6 ± 5.9	not tested
IL-17	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	not tested
IP-10 (CXCL-10)	n.d.	n.d.	2,863 ± 2,145	2,173 ± 687	13.2 ± 13.2	16,031 ± 6,808	*
MCP-1 (CCL-2)	n.d.	77.9 ± 55.0	249.1 ± 179.6	6.0 ± 1.4	349.5 ± 136.2	7,864 ± 2,984	*
Mip-1α (CCL-3)	n.d.	n.d.	3.8 ± 1.2	1,102 ± 593	n.d.	593.2 ± 240.6	n.s.
Mip-1β (CCL-4)	n.d.	n.d.	111.0 ± 68	7,169 ± 860	n.d.	8,122 ± 1,402	not tested
TNFα	n.d.	n.d.	26.7 ± 8.2	3,278 ± 1,179	n.d.	2,498 ± 966	*
VEGF	3.9 ± 0.4	210.9 ± 31.2	227.5 ± 76.9	n.d.	375.7 ± 36.5	250.6 ± 8.4	*
VCAM	131,879 ± 5,320	162,943 ± 5,306	141,916 ± 1,244	135,767 ± 0	144,771 ± 0	177,038 ± 0	not tested

4.3. Impact of BM-MSC on functionality of human mDC

A second type of DC that can readily be isolated from human blood are $CD1c^+$ (BDCA1) mDC (see paragraph 1.2.2.3), which produce high amounts of cytokines and chemokines [173,181] and are specialized to sense fungi, bacteria and injured tissue [58]. Therefore, we isolated *in vivo* generated, immature $CD1c^+$ mDC from human blood and analysed how BM-MSC influence their *in vitro* maturation by TLR ligands in terms of maturation

marker expression, CCR7-dependent migration, cytokine production and their ability to activate NK and naïve T cells.

4.3.1. BM-MSC have only minor effects on acquisition of maturation markers by *in vivo* differentiated mDC

As depicted in Figure 22A and similar to pDC *ex vivo*, freshly isolated mDC lacked expression of CD80, CD86, CD83 and CD40, but were highly positive for HLA-DR, a typical expression profile for immature DC. After activation by TLR ligands LPS/R848, mDC had acquired the expression of all analysed maturation markers (Figure 22). When mDC were matured in the presence of BM-MSC ([BM-MSC]mDC), we observed only a slightly, but significantly reduced expression of CD83 and CD40, while the costimulatory molecules CD80 and CD86 were even marginally upregulated and HLA-DR expression remained unchanged (Figure 22).

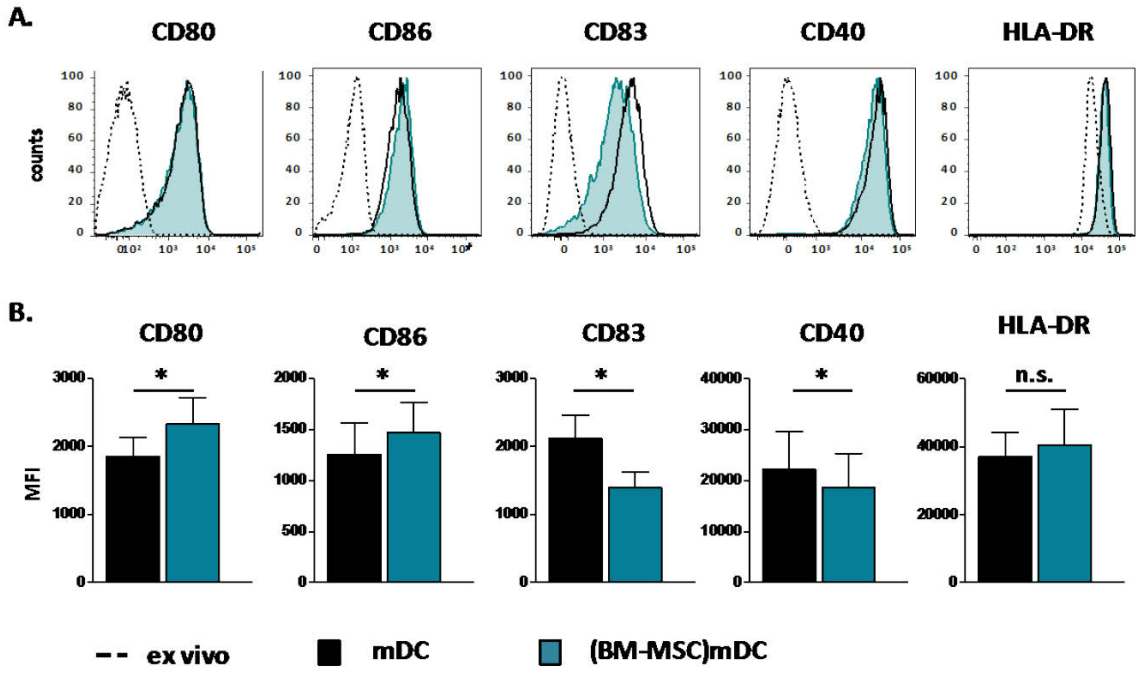

Figure 22: BM-MSC have only minor effects on maturation of *in vivo* generated mDC. (A-B) mDC were matured in the presence (blue) or absence (black) of BM-MSC by LPS/R848 for 18hrs. Expression of CD86, CD80, CD83, CD40 and HLA-DR by mDC was investigated by flow cytometry (n=7). **(A)** Histograms for mature mDC and (BM-MSC)mDC are shown for one representative donor. *Ex vivo* expression of the indicated markers by mDC are depicted as dotted lines. **(B)** Mean MFI values for the specified cell surface markers +/- SEM are presented for mDC alone or (BM-MSC)mDC. P<0.05 (*).

4.3.2. BM-MSC inhibit CCR7-dependent migration of mDC

Freshly isolated mDC did not express CCR7, as shown in Figure 23A. When matured by low dose LPS, approximately 60% of mDC acquired CCR7 expression (Figure 23A and Figure 23C). In the presence of BM-MSC, the percentage of CCR7^{high} mDC and the CCR7 level per cell, as measured by MFI of the CCR7^{high} mDC, were considerably decreased (Figure 23C). As a result, we observed a significant reduction of mDC that migrated towards the receptor's ligand CCL21 (Figure 23D).

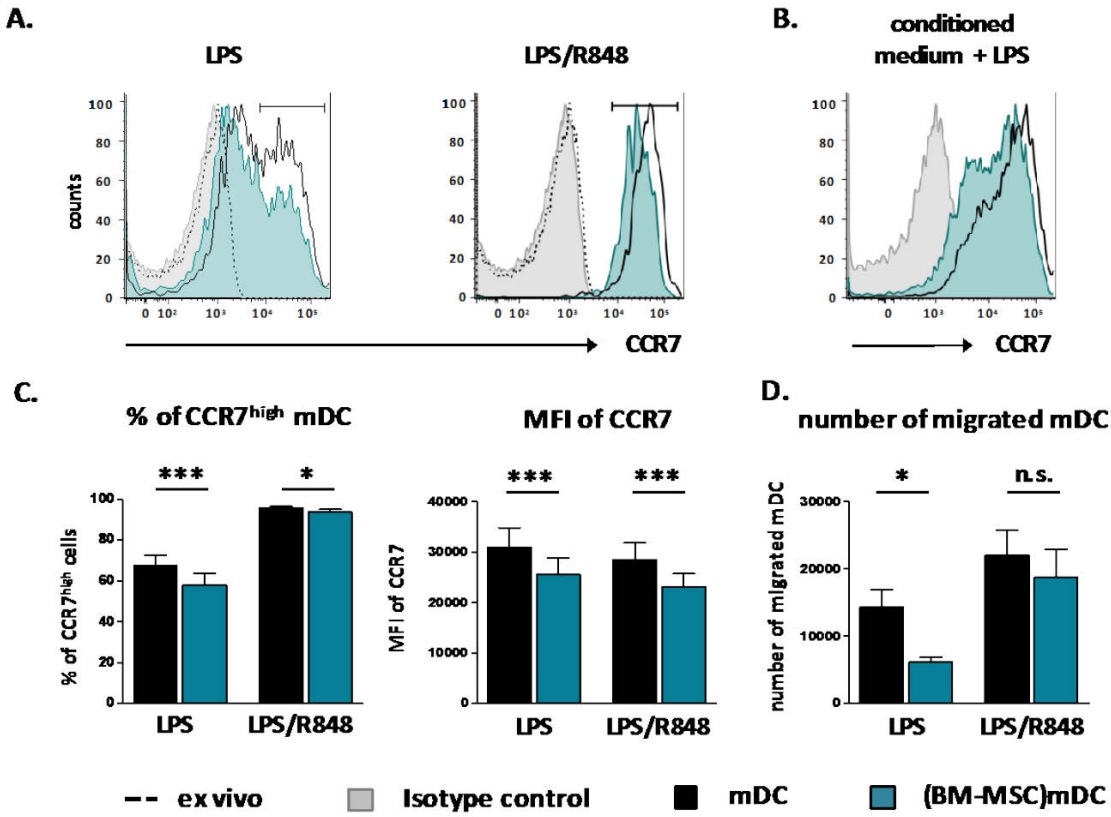

Figure 23: BM-MSC inhibit CCR7-dependent migration of mDC. (A-D) mDC were matured using low dose (100pg/ml LPS) or high dose (100ng/ml LPS+10µg/ml R848) TLR ligands for 18hrs. CCR7 expression was analysed by flow cytometry. Transwell migration assay was performed towards CCL21. **(A)** Data are shown for mDC (black), (BM-MSC)mDC (blue), *ex vivo* isolated mDC (dotted line) and the corresponding isotype control (light grey) for one representative donor. **(B)** mDC were matured in control or BM-MSC-conditioned medium supplemented by low dose LPS. Histograms are depicted for one representative donor out of three. BM-MSC were cultured in complete RPMI medium without TLR ligands for two days to generate BM-MSC conditioned medium. **(C)** The percentage of CCR7^{high} mDC and the MFI of CCR7^{high} mDC are presented as mean +/- SEM (n=13-15). **(D)** The number of migrated mDC is shown as mean +/- SEM (n=6-8). P<0.05 (*) and P<0.0005 (***).

To exclude that BM-MSC compete for low dose LPS during co-culture with mDC, BM-MSC-conditioned medium supplemented by LPS was used for mDC maturation. The

equal reduction of CCR7 expression (Figure 23B) shows firstly that the inhibitory effect of BM-MSC was mediated by soluble factors and did not depend on cell-cell contact. Secondly, it reveals that BM-MSC do not compete for LPS. Nevertheless, the inhibition could be overcome when mDC were activated using a combination of high dose LPS and R848, which is needed to efficiently induce cytokine production by mDC *in vitro* [173]. BM-MSC only slightly decreased CCR7 expression as well as migration towards CCL21 under these high dose stimulation conditions (Figure 23A-D).

4.3.3. BM-MSC shift the cytokine production of mDC towards an anti-inflammatory profile

The secretion profile of DC is import for the regulation of immune responses. To date, no extensive analysis about the influence of MSC on the DC secretome has been performed. Here, we investigated cytokine production of mDC in the presence or absence of BM-MSC by analysing culture supernatants by multiplex analysis.

A selection of cytokines produced in mDC cultures is shown in Figure 24, whereas the summary of all analytes is depicted in Table 12. When freshly isolated immature mDC were cultured in medium without TLR ligands, they produced almost no cytokines. Consistent with the current literature [173], mDC needed high dose stimulation by using a combination of LPS and R848 for efficient cytokine secretion. Compared to mDC alone, we measured significantly less pro-inflammatory cytokines IL-12p70 and IL-1β in mDC/BM-MSC co-cultures upon activation by LPS/R848 (Figure 24A). In contrast, there was a considerable increase of the anti-inflammatory cytokines IL-10 as well as IL-1Ra. Moreover, IL-6 production was significantly enhanced. By calculating the ratios between pro-inflammatory IL-12 and anti-inflammatory IL-10 as well as pro-inflammatory IL-1β and anti-inflammatory IL-1Ra (Figure 24B), we could show that BM-MSC altered the secretion profile of mDC towards an anti-inflammatory profile. Other cytokines, such as IFNγ or TNFα were not regulated by BM-MSC (Table 12).

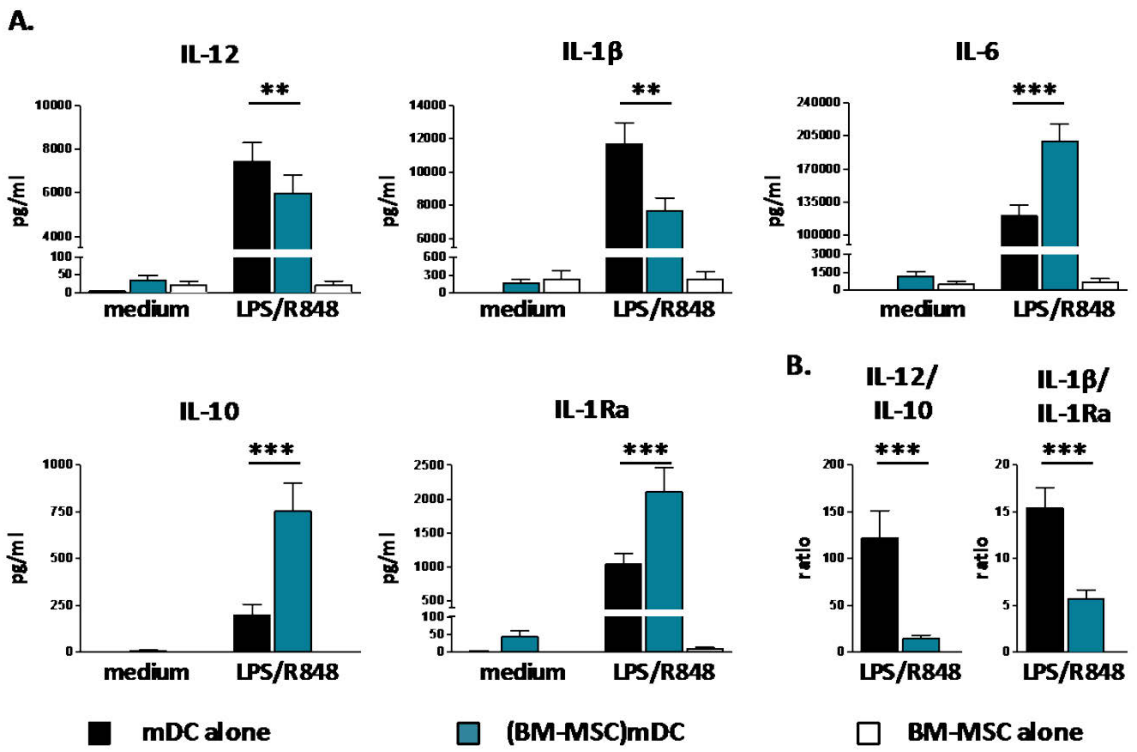

Figure 24: BM-MSC shift the cytokine production of mDC towards an anti-inflammatory profile. (A-B) Cytokine production in mDC/BM-MSC co-culture supernatants was measured by multiplex analysis (n=21). mDC alone (black), (BM-MSC)mDC (blue) or BM-MSC alone (white) were cultured in medium with or without LPS/R848 for 18hrs. **(A)** Concentrations of the indicated cytokines are depicted as mean +/- SEM. **(B)** Ratios between IL-12 and IL-10 as well as IL-1β and IL-1Ra are presented as mean +/- SEM. P<0.005 (**) and P<0.0005 (***).

Table 12: Analysis of cytokines in mDC/BM-MSC co-culture supernatants. Cytokine levels in mDC/BM-MSC co-culture supernatants were determined by multiplex analysis (n=21). mDC alone, (BM-MSC)mDC or BM-MSC alone were cultured in medium with or without LPS/R848 for 18hrs. Concentrations of the respective cytokines are presented as mean +/- SEM (pg/ml). Significance was tested between LPS/R848-matured mDC and mDC/BM-MSC co-cultures. *Abbreviations: ctr., control; n.d., not detectable; n.s., not significant, P<0.005 (**) and P<0.0005 (***).*

Analyte	mDC ctr. (pg/ml)	BM-MSC ctr. (pg/ml)	mDC/BM-MSC ctr. (pg/ml)	mDC LPS/R848 (pg/ml)	BM-MSC LPS/R848 (pg/ml)	mDC/BM-MSC LPS/R848 (pg/ml)	Significance
IFNγ	n.d.	n.d.	n.d.	406.2 ± 34.3	n.d.	409.8 ± 35.2	not tested
IL-1β	n.d.	229.8 ± 146.7	159.5 ± 69.9	11,633 ± 1,336	218 ± 142	7,687 ± 755	**
IL-1ra	2.5 ± 1.8	n.d.	43.6 ± 17.9	1,034 ± 159	8.1 ± 5.6	2,106 ± 354	***
IL-2	n.d.	n.d.	n.d.	54.1 ± 9.8	n.d.	55.6 ± 9.4	not tested
IL-4	n.d.	n.d.	n.d.	22.2 ± 4.3	n.d.	23.2 ± 4.3	not tested
IL-5	n.d.	n.d.	n.d.	1.4 ± 0.7	n.d.	1.7 ± 0.9	not tested
IL-6	1.1 ± 0.5	467.7 ± 236.6	1.143 ± 408	119,924 ± 12,055	652.3 ± 301.6	199,517 ± 18,232	***
IL-7	n.d.	1.3 ± 0.8	2.4 ± 1.2	3.3 ± 1.7	1.3 ± 0.8	4.3 ± 2.3	not tested
IL-9	2.0 ± 1.0	2.7 ± 1.9	11.5 ± 5.4	65.2 ± 29.3	3.2 ± 2.2	73.6 ± 32.9	n.s.
IL-10	1.9 ± 0.8	2.2 ± 1.3	6.8 ± 3.4	200.7 ± 56.4	1.5 ± 0.9	754.8 ± 148.8	***

Analyte	mDC ctr. (pg/ml)	BM-MSC ctr. (pg/ml)	mDC/BM-MSC ctr. (pg/ml)	mDC LPS/R848 (pg/ml)	BM-MSC LPS/R848 (pg/ml)	mDC/BM-MSC LPS/R848 (pg/ml)	Significance
IL12p40	n.d.	n.d.	n.d.	149,635 ± 31,631	n.d.	135,434 ± 18,612	not tested
IL-12p70	3.3 ± 1.2	21.1 ± 10.2	35.1 ± 13.3	7,439 ± 862	22.3 ± 10.6	5,993 ± 827	**
IL-13	1.2 ± 0.6	1.7 ± 1.1	3.2 ± 1.7	1.7 ± 0.9	1.7 ± 1.1	3.3 ± 1.9	not tested
IL-15	1.2 ± 0.6	n.d.	n.d.	11.4 ± 2.8	1.1 ± 0.7	13.4 ± 2.4	n.s.
IL-17	n.d.	1.6 ± 1.6	2.7 ± 1.7	100.4 ± 42.0	3.4 ± 3.4	106.3 ± 44.6	n.s.
IL-18	2.0 ± 1.7	n.d.	1.6 ± 1.4	64.3 ± 10.2	2.2 ± 2.0	56.2 ± 7.3	n.s.
TNFα	16.0 ± 7.6	1.6 ± 1.1	17.6 ± 4.9	104,443 ± 17,873	2.3 ± 1.3	101,131 ± 20,230	n.s.

4.3.4. mDC matured in the presence of BM-MSC show a reduced ability for NK cell activation

The crosstalk between DC, NK and T cells is very important for the initiation of immune responses (see section 1.2.5). DC-derived cytokines induce activation of both NK and T cells [54,60], while NK cell-derived IFNγ is known to be essential for Th1 lineage commitment [103,126]. In order to investigate if BM-MSC modulate the capacity of mDC for NK cell activation, we matured mDC in the presence or absence of BM-MSC by TLR ligands and used these mDC in a second step to activate sorted $CD56^{bright}$ or $CD56^{dim}$ NK cells (see paragraph 3.2.14.1). NK cell activation was characterized by their proliferation rate, cytotoxic capacity and IFNγ production.

4.3.4.1. BM-MSC-licensed mDC induce less NK cell proliferation

Upon cytokine stimulation, $CD56^{bright}$ NK cells display a higher proliferative capacity than the $CD56^{dim}$ compartment [96]. In line with that, mDC induced higher proliferation in $CD56^{bright}$ than in $CD56^{dim}$ NK cells, as measured by CFSE dilution after five days of co-culture (Figure 25). In comparison, activation by (BM-MSC)mDC led to a lower proliferation rate of both NK cell subsets.

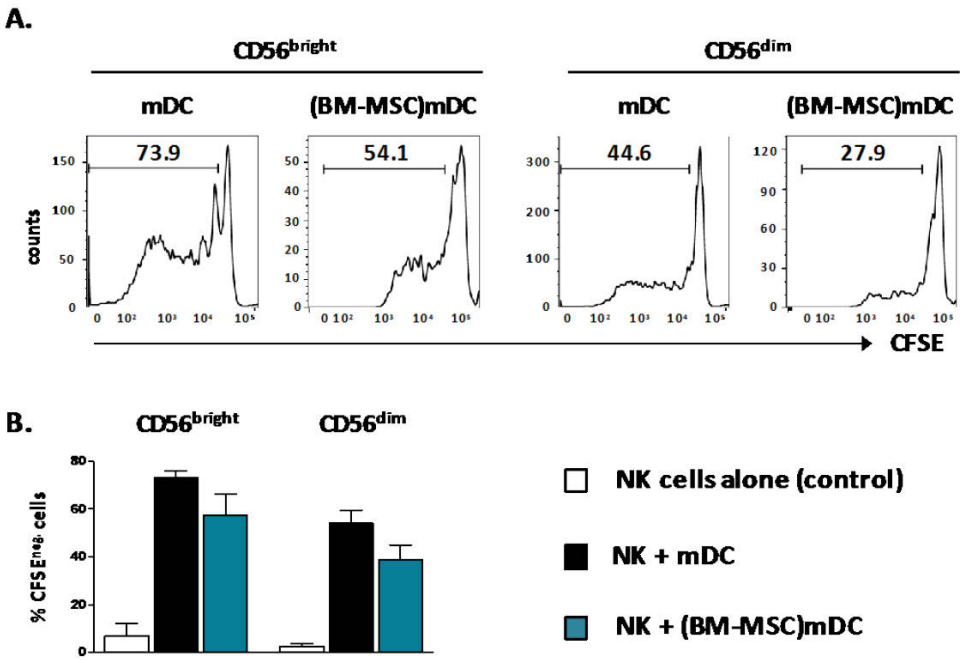

Figure 25: BM-MSC-licensed mDC induce less NK cell proliferation. (A-B) Sorted CD56^{bright} or CD56^{dim} NK cells were cultured alone (white) or together with TLR ligand matured mDC (black) or (BM-MSC)mDC (blue). Proliferation of NK cell subsets was measured by CFSE dilution after five days of co-culture with mDC. **(A)** Histograms for one representative donor out of five are shown. **(B)** Mean percentage of CFSE^{-} cells +/- SEM are depicted.

4.3.4.2. BM-MSC-licensed mDC display a reduced ability to enhance the cytotoxic potential of CD56^{dim} NK cells

While CD56^{bright} NK cells are not cytotoxic, the cytotoxic CD56^{dim} NK cell compartment even increases its cytotoxic capacity when stimulated by DC-derived cytokines [96]. Therefore, we preactivated CD56^{dim} NK cells by mDC or (BM-MSC)mDC, before analysing their cytotoxic potential towards the MHC class I negative tumour target cells K562. This was measured by CD107a mobilization and specific killing of the target cells K562, a marker for degranulation, on NK cells.

Compared to resting NK cells, the cytotoxic potential of CD56^{dim} NK cells towards target cells K562 was increased after short term preactivation by mDC, as reflected by CD107a expression on NK cells (Figure 26A) as well as specific killing of target cells K562 (Figure 26B). In contrast, preactivation by (BM-MSC)mDC was slightly less efficient in enhancing CD107a expression by NK cells as well as specific killing of K562 cells.

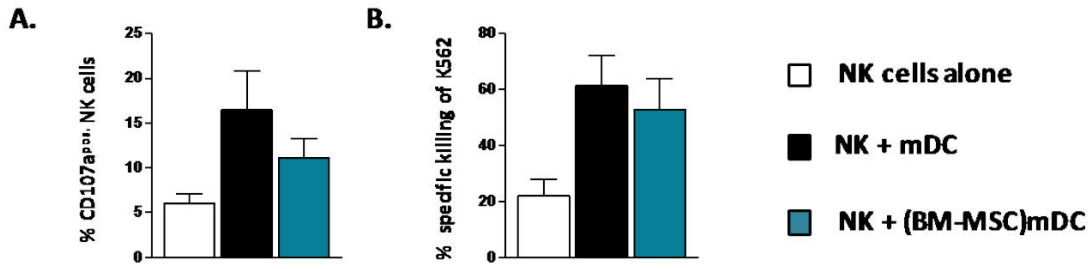

Figure 26: BM-MSC-licensed mDC display a reduced ability to enhance the cytotoxic potential of CD56^{dim} NK cells. (A-B) CD56^{dim} NK cells were preactivated by mature mDC (black) or (BM-MSC)mDC (blue) for 18hrs before being stimulated with K562 cells for 6hrs. Resting NK cells served as control (white). CD107a expression on NK cells **(A,** n=3**)** and specific killing of K562 cells **(B,** n=4**)** are presented as mean +/- SEM.

4.3.4.3. BM-MSC-licensed mDC induce less IFNγ production in NK cells

NK cell-derived IFNγ has been shown to sustain Th1 priming and therefore to be indispensable for the crosstalk between DC, NK and T cells. We analysed IFNγ production in CD56^{dim} as well as CD56^{bright} NK cells with the latter being the main IFNγ producers upon cytokine stimulation.

When activated by mDC for 24hrs, approximately 30% of CD56^{bright} NK cells produced IFNγ, whereas only around 6% of CD56^{dim} NK cells were IFNγ^{+}(Figure 27A and Figure 27B). We observed a significantly decreased IFNγ production in both CD56bright and CD56^{dim} NK cells when they were stimulated by (BM-MSC)mDC. This could be seen by a strongly reduced percentage of IFNγ^{+} cells and a decreased amount of IFNγ produced per cell, as measured by MFI of the IFNγ^{+} cells.

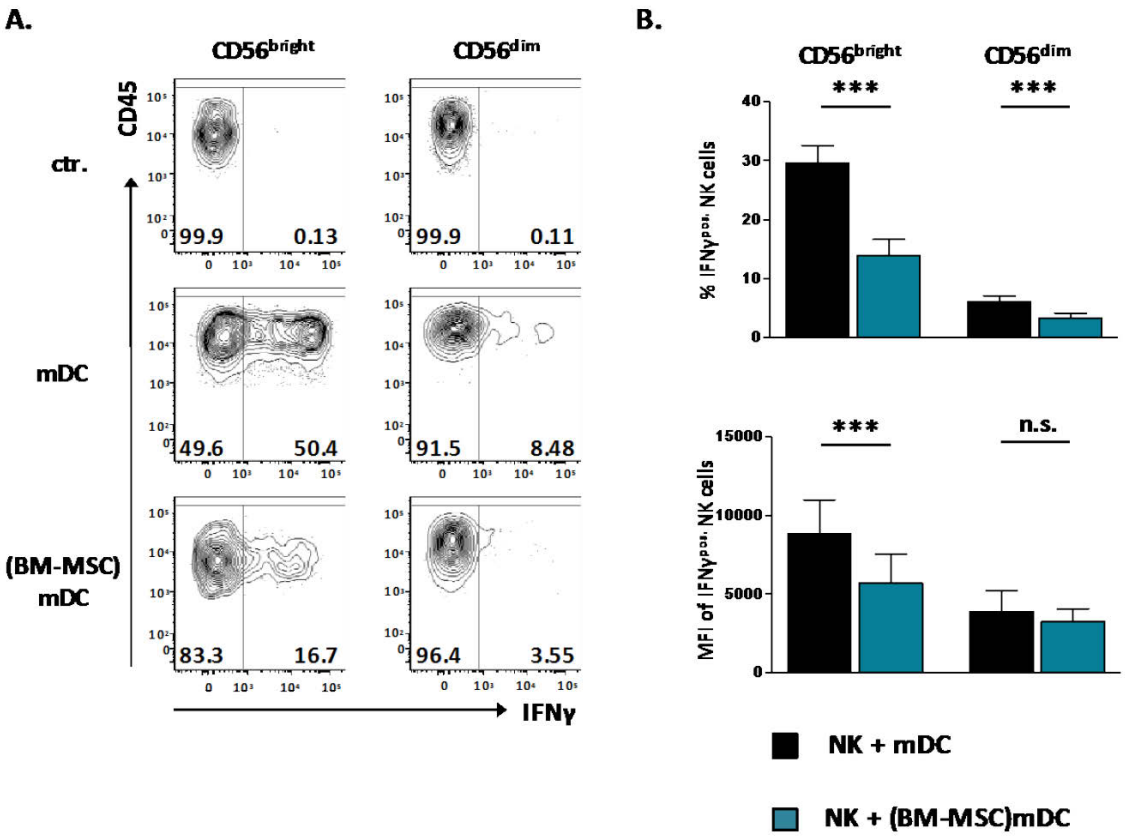

Figure 27: BM-MSC-licensed mDC induce less IFNγ production in NK cells. (A-B) Mature mDC (black) or (BM-MSC)mDC (blue) were used to activate sorted CD56^{bright} or CD56^{dim} NK cells for 24hrs (n=11-15). Resting NK cells were used as control. IFNγ production was assessed by intracellular staining and analysed by flow cytometry. **(A)** Data are shown for one representative donor. **(B)** Percentage of IFNγ^{+} NK cells as well as MFI values of IFNγ^{+} cells are depicted as mean +/- SEM. P<0.0005 (***).

4.3.5. The diminished ability of BM-MSC-licensed mDC to activate NK cells is a result of their increased IL-10 production

The inhibitory effects of MSC on DC functionality seem to be mainly dependent on soluble factors [143]. Nevertheless, some authors reported at least a partial role for contact-dependent mechanisms [146,151,160]. By using BM-MSC-conditioned medium, we could show that the inhibitory effect of BM-MSC on CCR7 expression by mDC was mediated by soluble factors (see section 4.3.2, Figure 23). Therefore, we performed transwell experiments to confirm the role of soluble factors. Moreover, we blocked different interesting soluble factors in mDC/BM-MSC co-cultures as well as in mDC/NK cell co-cultures to build a more detailed picture on the molecular mechanism behind the inhibitory effects of BM-MSC.

4.3.5.1. IL-10 is the key player regulating the altered cytokine production by BM-MSC-licensed mDC

Since (BM-MSC)mDC produced higher levels of IL-10, IL-1Ra and IL-6, which were shown before to play an immunomodulatory role [182,183,184], we wanted to understand if these cytokines regulate the expression of pro-inflammatory cytokines by mDC. We blocked IL-10, IL-1Ra and IL-6 by neutralizing antibodies during the mDC/BM-MSC co-culture and analysed the cytokine levels by multiplex analysis.

As shown in Figure 28, the detectable levels of IL-6 and IL-10 were strongly reduced under neutralizing conditions, whereas IL-1Ra could still be quantified in the presence of neutralizing anti-IL-1Ra. Most likely, the neutralizing and detection antibody for IL-1Ra recognized distinct epitopes, while the same epitopes were recognized by neutralizing and detection antibodies for IL-10/IL-10R and IL-6. For this reason, it was impossible to specifically measure biologically active IL-1Ra under neutralizing conditions, while it could be clearly demonstrated that biologically active IL-6 and IL-10 were absent in the presence of the respective blocking antibodies. However, the antibody used for blocking of IL-1Ra was functional as shown by the effect on the production of other cytokines (e.g. IL-12 or IL-1β, Figure 29).

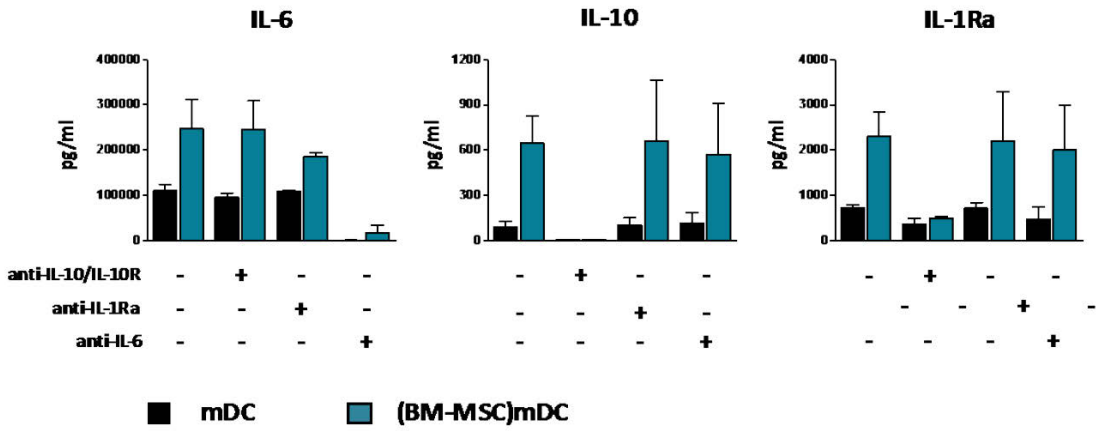

Figure 28: Levels of IL-6, IL-10 and IL-1Ra under neutralizing conditions in mDC/BM-MSC co-cultures. Cytokine concentrations in LPS/R848 matured mDC/BM-MSC co-culture supernatants were determined by multiplex analysis (n=3-5) and are shown as mean +/- SEM. mDC alone (black) or (BM-MSC)mDC (blue) were cultured in medium supplemented by LPS/R848 and neutralizing antibodies for IL-10/IL-10R, IL-1Ra or IL-6 as indicated.

The IL-12p70 level could be restored by applying neutralizing antibodies for IL-10/IL-10R, IL-1Ra or IL-6 in mDC/BM-MSC co-cultures, while IL-1β production could only partially be

reconstituted by blocking these three factors (Figure 29). The level of IL-1Ra could only be diminished in the presence of anti-IL-10/anti-IL-10R, indicating that IL-1Ra production is regulated by IL-10. Moreover, the increased levels of IL-10 could exclusively be decreased by blocking IL-10/IL-10R, but not by neutralization of IL-1Ra or IL-6.

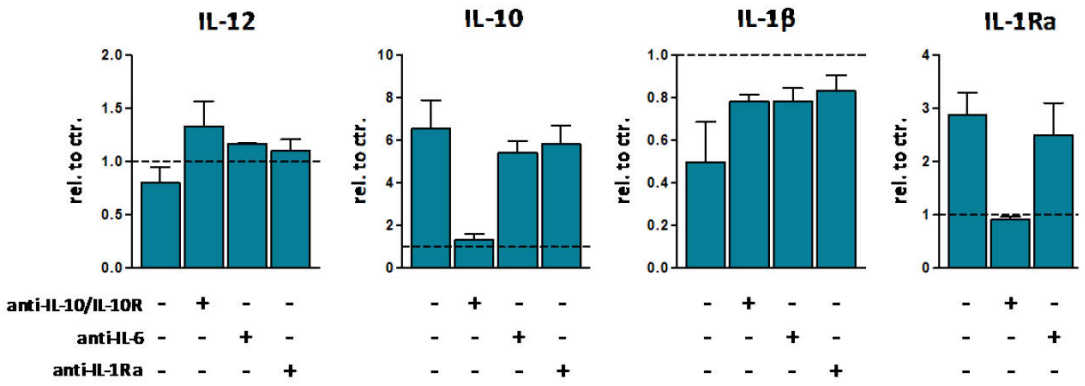

Figure 29: IL-10 regulates the altered cytokine production by (BM-MSC)mDC. mDC alone (black) or (BM-MSC)mDC (blue) were matured in the presence of neutralizing antibodies for IL-10/IL-10R, IL-1Ra or IL-6 as indicated for 18hrs. Cytokine levels of mDC/BM-MSC co-culture supernatants were measured by multiplex analysis (n=3-5) and normalized to mDC alone (dashed line). Results are depicted as mean +/- SEM.

In conclusion, we observed that the three analysed factors (IL-10, IL-1Ra and IL-6) regulated IL-12 as well as IL-1β production to the same extent (Figure 29). However, IL-10 was the only factor also regulating IL-1Ra expression, which indicates its importance for the regulation of cytokine production by mDC. Indeed, by calculating the ratios between IL-12 and IL-10 as well as IL-1β and IL-1Ra (Figure 30), we could show that only neutralization of IL-10/IL-10R normalized the balance between pro- and anti-inflammatory cytokines in the presence of BM-MSC. In summary, it can be said that the balance between pro- and anti-inflammatory cytokines produced by mDC was mainly regulated by the level of biologically active IL-10.

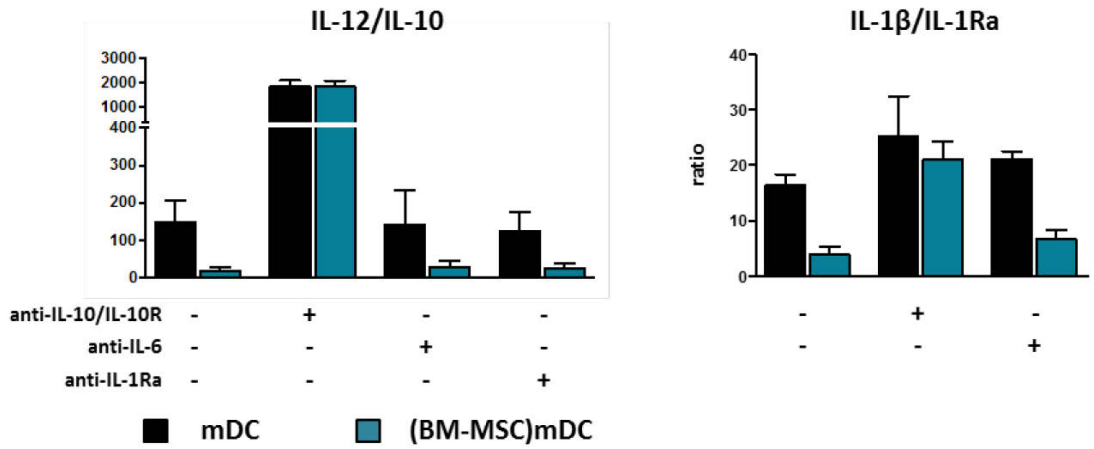

Figure 30: IL-10 is the key player for the altered cytokine production by (BM-MSC)mDC. mDC alone (black) or (BM-MSC)mDC (blue) were stimulated in the presence of blocking antibodies for IL-10/IL-10R, IL-1Ra or IL-6 as specified for 18hrs. Cytokine production of mDC/BM-MSC co-cultures were analysed by multiplex analysis of supernatants (n=3-5). Ratios between IL-12 and IL-10 as well as IL-1β and IL-1Ra are presented as mean +/- SEM.

4.3.5.2. The decreased IL-12 production by (BM-MSC)mDC accounts for the reduced NK cell stimulation

DC can induce IFNγ production in NK cells either by secretion of pro-inflammatory cytokines, such as IL-12, IL-15 or IL-18, or by ligation of activating receptors on NK cells [127,185]. In order to understand which of the two mechanisms prevailed in our culture, we co-cultured mDC and $CD56^{bright}$ NK cells in transwell plates, either separated by the membrane or in contact in the lower chamber. In both cases, the percentage of $IFN\gamma^{+}$ NK cells was comparable (Figure 31A), showing that NK cell activation was mediated by DC-derived cytokines. Moreover, the reduced ability of (BM-MSC)mDC to stimulate NK cells could still be observed in the transwell setting. This suggests that (BM-MSC)mDC either secreted less of an activating or an additional inhibitory factor. Therefore, we wanted to understand if the altered cytokine profile of (BM-MSC)mDC (see paragraph 4.3.3, Figure **24**) was responsible for their diminished ability to induce IFNγ production in NK cells. When we neutralized IL-12 in mDC/NK cell co-cultures, NK cells did not produce IFNγ anymore, showing the crucial role of this cytokine for NK cell activation in our setting (Figure 31C). Additionally, by adding recombinant IL-12 to the (BM-MSC)mDC/NK cell culture, we could completely reconstitute the IFNγ production (Figure 31B and Figure 31C). In contrast, the neutralization of IL-10/IL-10R, IL-1Ra or IL-6 in mDC/NK cell co-cultures had no effect on the percentage of IFNγ producing NK cells (Figure 31B and

Figure 31C). In summary, it can be concluded that the lower IL-12 production by (BM-MSC)mDC was responsible for the decreased IFNγ level in NK cells while IL-10, IL-1Ra or IL-6 had no direct effect on NK cell activation.

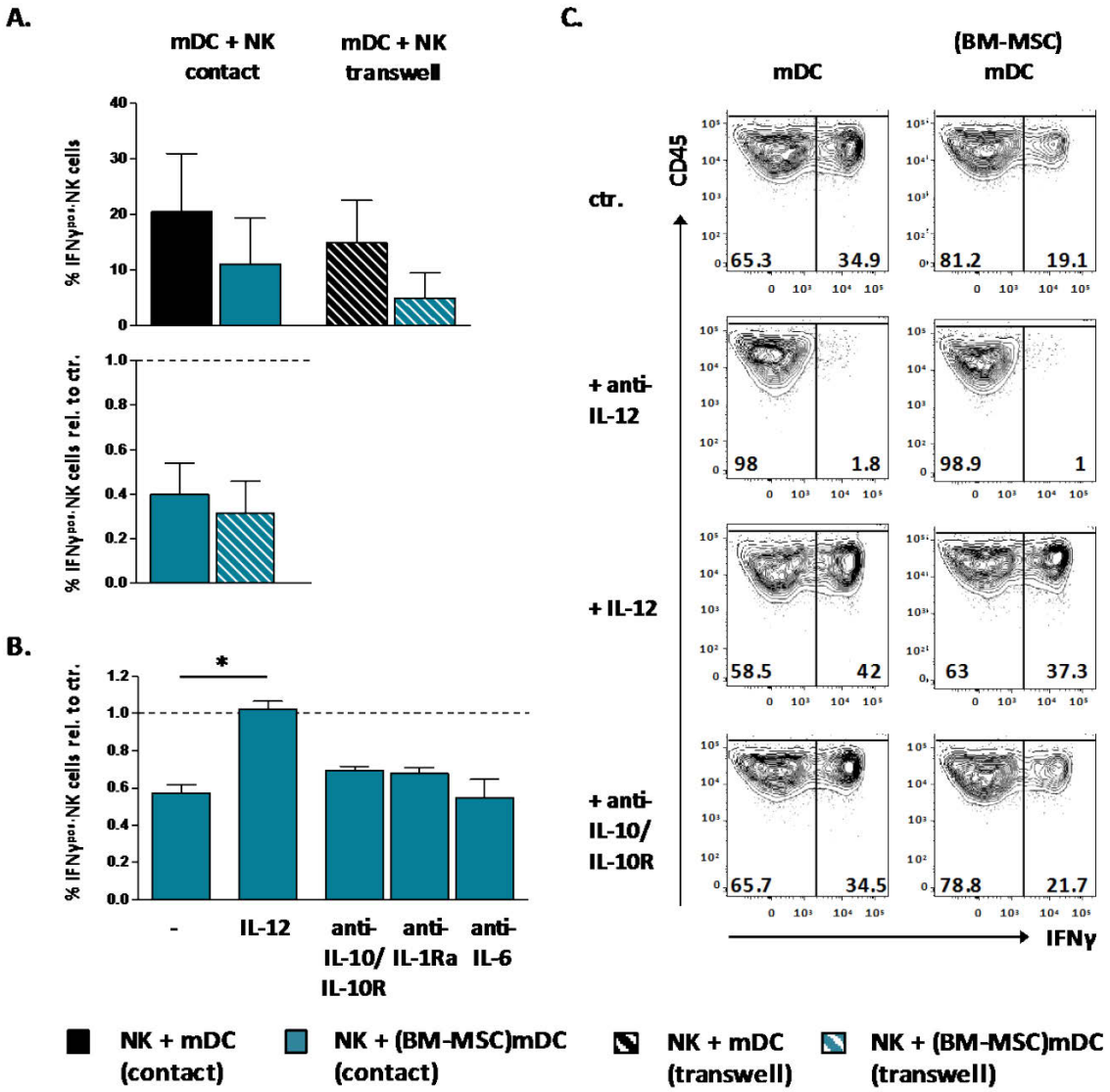

Figure 31: Decreased IL-12 production by (BM-MSC)mDC accounts for the reduced NK cell stimulation. **(A-C)** TLR ligand-activated mDC (black) or (BM-MSC)mDC (blue) were co-cultured with CD56^{bright} NK cells for 24hrs before intracellular staining for IFNγ. **(A)** mDC and NK cells were seeded in transwell plates, either in contact in the lower chamber (solid bars) or separated by the membrane (striped bars). Mean percentage of IFNγ^{+} NK cells (upper graph) and mean percentage of IFNγ^{+} NK cells relative to stimulation with control mDC matured in the absence of BM-MSC (dashed line) +/- SEM (lower graph) are depicted (n=3). **(B-C)** Recombinant IL-12 or blocking antibodies for IL-10/IL-10R, IL-1Ra or IL-6 were added during NK cell stimulation as indicated (n=3-8). Mean percentage of IFNγ^{+} NK cells in relation to control without BM-MSC (dashed line) +/- SEM **(B)** and representative data for one donor **(C)** are shown. P<0.05 (*).

4.3.5.3. IL-10 regulates mDC's ability to induce IFNγ production in NK cells

Next, we wanted to investigate how BM-MSC diminished the ability of mDC to stimulate NK cells. For this reason, mDC and BM-MSC were cultured in the transwell setting to exclude contamination with BM-MSC during NK cell culture. mDC were used in a second step for NK cell activation. As shown in Figure 32A, BM-MSC did not need to be in direct contact with mDC to reduce their capacity for induction of IFNγ production in NK cells. Here again, the inhibitory rate of BM-MSC was comparable in the contact and transwell settings, indicating that a soluble factor triggered this effect.

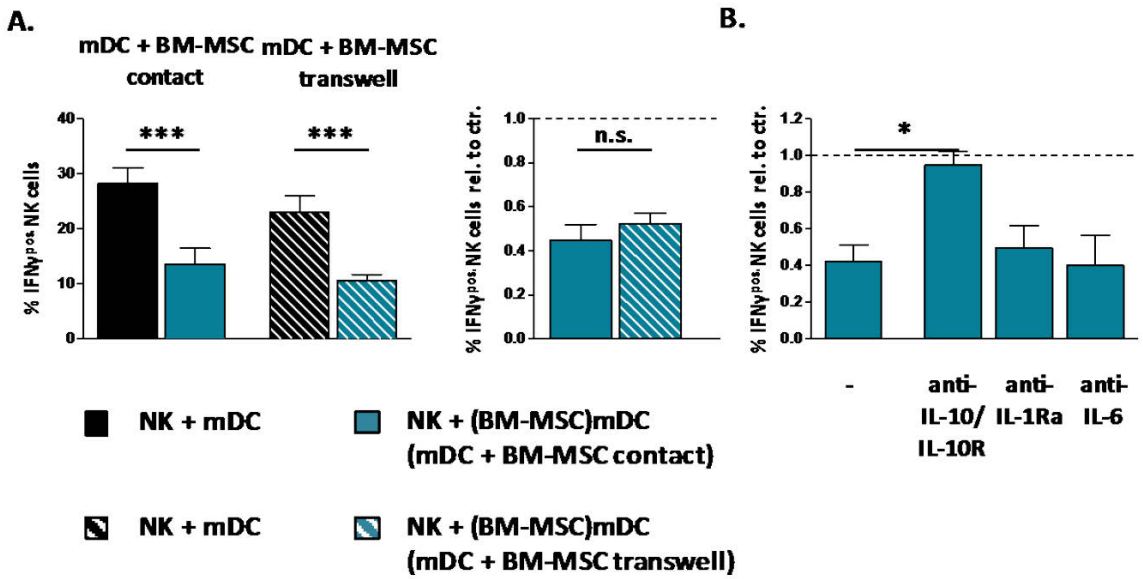

Figure 32: IL-10 regulates the ability of mDC to induce IFNγ production in NK cells. (A-B) $CD56^{bright}$ NK cells were activated by mature mDC (black) or (BM-MSC)mDC (blue) for 24hrs before intracellular staining for IFNγ was analysed by flow cytometry. **(A)** mDC and BM-MSC were cultured in contact (solid bars) or separated by a transwell membrane (striped bars) before co-culture with NK cells (n=11-18). Mean percentage of $IFN\gamma^+$ NK cells (left graph) and mean percentage of $IFN\gamma^+$ NK cells relative to control without BM-MSC (dashed line, right graph) +/- SEM are shown. **(B**) Blocking antibodies for IL-10/IL-10R, IL-1Ra or IL-6 were added during mDC stimulation (n=6). Percentage of $IFN\gamma^+$ NK cells were normalized to control mDC that have been matured without BM-MSC, but in the presence of the indicated neutralizing antibodies (dashed line). Results are presented as mean +/- SEM. $P<0.05$ (*) and $P<0.0005$ (***).

Because we observed that IL-12 production of mDC was regulated by IL-10, IL-1Ra and IL-6 (see section 4.3.5.1, Figure 29), we wanted to understand if one of these factors is the key player for the observed inhibitory effect on NK cell activation. While none of these factors had a direct effect during NK cell activation (see paragraph 4.3.5.2, Figure 31), the addition of neutralizing antibodies for IL-10/IL-10R to the mDC/BM-MSC co-culture system completely restored the ability of (BM-MSC)mDC to induce IFNγ

production in NK cells (Figure 32B). In contrast, blocking of IL-1Ra or IL-6 in mDC/BM-MSC co-cultures did not reconstitute the IFNγ level in NK cells (Figure 32B).

In summary, we can conclude that by increasing IL-10 production of mDC, BM-MSC shifted their cytokine profile towards an anti-inflammatory status characterized among others by low IL-12 production, which is the reason for a diminished ability of (BM-MSC)mDC to activate NK cells.

4.3.6. mDC secrete higher levels of growth factors and chemokines in the presence of BM-MSC

Chemokines and growth factors are other important factors that are secreted by mDC and can influence a broad range of cells. We studied the influence of BM-MSC on cytokine secretion by mDC by performing a multiplex assay of culture supernatants. A selection of chemokines and growth factors is depicted in Figure 33, while the summary of all analytes can be found in Table 13.

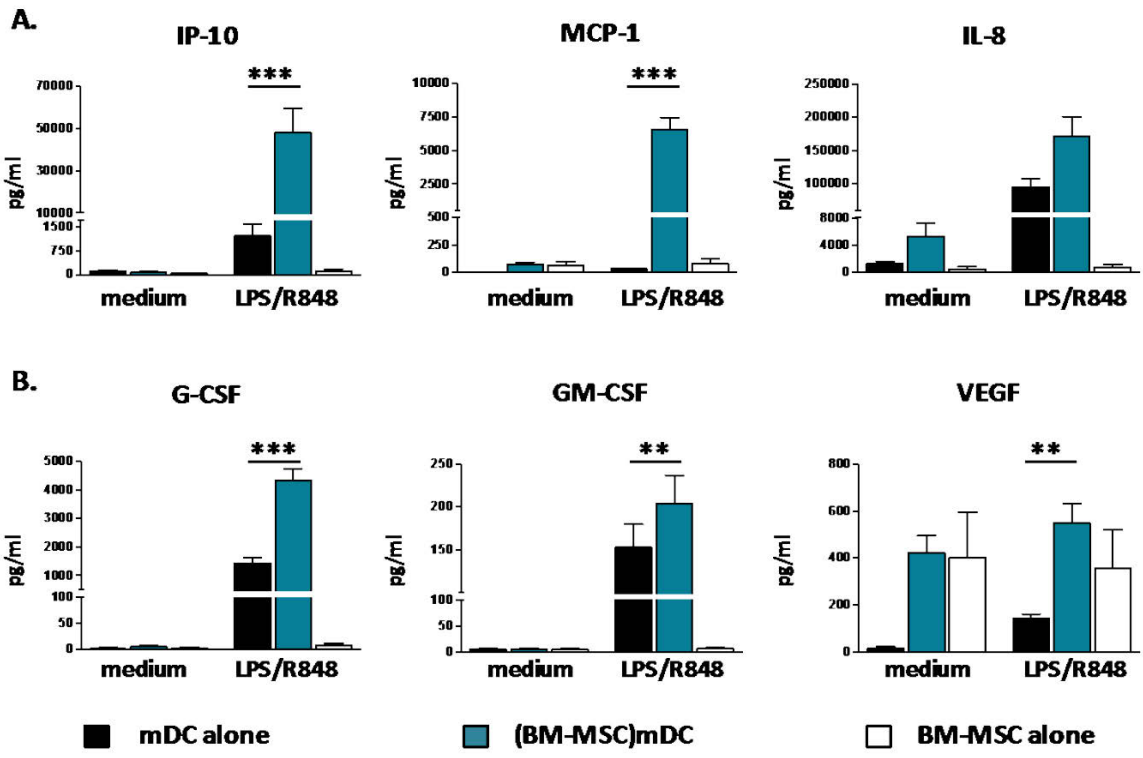

Figure 33: Increased production of growth factors and chemokines mDC/BM-MSC co-cultures. (A-B) Production of growth factors and chemokines in mDC/BM-MSC co-culture supernatants was determined by multiplex analysis (n=21). mDC alone (black), (BM-MSC)mDC (blue) or BM-MSC alone (white) were cultured in medium with or without LPS/R848 for 18hrs. Concentrations of the indicated growth factors and chemokines are shown as mean +/- SEM. P<0.005 (**) and P<0.0005 (***).

We detected significantly increased levels for IP-10 and MCP-1 in mDC/BM-MSC co-cultures (Figure 33A). The secretion of the chemokine IL-8 was also strongly increased, but this effect was not significant. Furthermore, the secretion of the growth factors granulocyte colony-stimulating factor (G-CSF), GM-CSF and VEGF was significantly higher in stimulated mDC/BM-MSC co-cultures compared to mDC alone (Figure 33B).

Table 13: Analysis of chemokines and growth factors in mDC/BM-MSC co-culture supernatants. Levels of the respective growth factors and chemokines in mDC/BM-MSC co-culture supernatants were determined by multiplex analysis (n=21). mDC alone, (BM-MSC)mDC or BM-MSC alone were cultured in medium with or without LPS/R848 for 18hrs. Concentrations of the respective cytokines are presented as mean +/- SEM (pg/ml). Significance was tested between LPS/R848 matured mDC and (BM-MSC)mDC. *Abbreviations: ctr., control; n.d., not detectable; n.s., not significant, P<0.005 (**) and P<0.0005 (***).*

Analyte	mDC ctr. (pg/ml)	BM-MSC ctr. (pg/ml)	mDC/BM-MSC ctr. (pg/ml)	mDC LPS/R848 (pg/ml)	BM-MSC LPS/R848 (pg/ml)	mDC/BM-MSC LPS/R848 (pg/ml)	Significance
bFGF	n.d.	8.7 ± 5.7	7.0 ± 4.6	121.5 ± 27.9	8.6 ± 6.9	125.6 ± 29.1	n.s.
Eotaxin	n.d.	n.d.	n.d.	145.2 ± 16.6	n.d.	148.6 ± 18.1	not tested
G-CSF	1.8 ± 1.8	2.9 ± 1.9	5.2 ± 2.6	1,440 ± 194	7.6 ± 3.2	4,317 ± 413	***
GM-CSF	5.3 ± 2.2	4.7 ± 2.6	5.1 ± 2.3	153 ± 26.4	6.3 ± 2.7	203.8 ± 32.7	**
IL-8	1,250 ± 329	458.8 ± 328.4	5,204 ± 2,048	86,724 ± 10,634	726 ± 356	145,145 ± 27,122	n.s.
IP-10 (CXCL-10)	101.9 ± 48.8	32.8 ± 20.5	62.2 ± 37.5	1,212 ± 387	107.8 ± 64.0	47,822 ± 11,488	***
MCP-1 (CCL-2)	1.3 ± 0.6	61.4 ± 36.6	66.2 ± 23.7	30.4 ± 4.0	80.7 ± 41.5	6,573 ± 866	***
MCP-3 (CCL-7)	n.d.	n.d.	n.d.	10.0 ± 4.9	n.d.	114.5 ± 21.2	not tested
Mip-1α (CCL-3)	26.3 ± 23.1	1.2 ± 0.2	1.2 ± 0.2	20,795 ± 4,407	1.4 ± 0.2	20,236 ± 1,944	not tested
Mip-1β (CCL-4)	237.7 ± 92.3	3.6 ± 3.6	247.4 ± 163.8	257,447 ± 70,383	15.4 ± 6.8	305,239 ± 113,415	n.s.
PDGF-bb	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	not tested
Rantes (CCL-5)	n.d.	n.d.	n.d.	2,033 ± 392	n.d.	2,907 ± 713.5	not tested
VEGF	16.2 ± 7.7	401.9 ± 192.7	423.4 ± 71.2	145.1 ± 16.9	354.8 ± 166.2	548.3 ± 81.3	**
VCAM	150.3 ± 7.0	162.9 ± 5.3	154.7 ± 5.0	139.3 ± 1.4	162.9 ± 18.7	149.3 ± 5.8	not tested

4.3.7. BM-MSC-licensed mDC display a reduced capacity to induce Th1 priming *in vitro*

Beside NK cells, DC also encounter and activate naïve T cells in the lymph node. As described above, BM-MSC shifted the cytokine production by mDC towards an anti-inflammatory profile (see paragraph 4.3.3, Figure 24), whereas they did not have a clear

impact on the upregulation of costimulatory molecules (see section 4.3.1, Figure 22). Therefore, we analysed if (BM-MSC)mDC display an altered potential to induce lineage decision of allogeneic naïve T cells towards the Th1 compartment. Using CBA, we measured IFNγ levels in the culture supernatants after five days of mDC/T cell co-culture.

As shown in Figure 34, (BM-MSC)mDC induced less Th1 priming of allogeneic naïve T cells *in vitro*, as measured by reduced IFNγ levels in the culture supernatants. Additionally, this shows that allogeneic BM-MSC were not immunogenic by themselves since they did not enhance the response of allogeneic T cells towards the mDC.

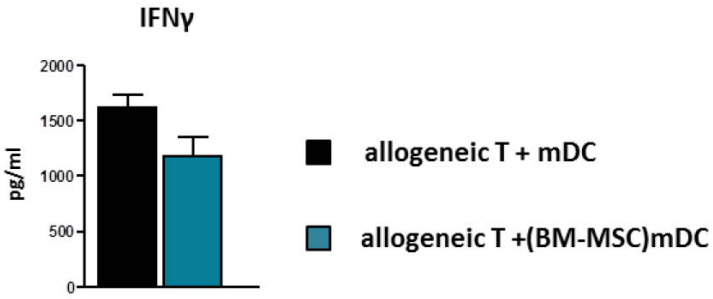

Figure 34: BM-MSC-licensed mDC display a reduced capacity to induce Th1 priming. Mature mDC (black) or (BM-MSC)mDC (blue) were used to stimulate allogeneic naïve T cells for five days. IFNγ levels in the culture supernatants were analysed by CBA and are presented as mean +/- SEM (n=4).

4.4. Immunomodulation of MSC-like PLX-PAD cells *in vivo*

There are preclinical and clinical trials showing that the use of allogeneic MSC is safe [37,38]. However, there exist also studies presenting clear hints from animal studies that MSC indeed can cause alloimmunity and even stimulate graft rejection [39,40,41]. Especially, there are no adequate immunological data from patients available until now. Our observation that MSC inhibit the migration of DC towards the lymph nodes as well as the crosstalk between DC, NK and T cells could be an explanation for the described inhibition of Th1-driven immune-pathological processes by MSC *in vivo,* such as GvHD or MS (see section 1.1.2) [162,186,187]. We had the chance to investigate samples from a phase I/IIa clinical trial, in which CLI patients were treated with allogeneic HLA-unmatched PLX-PAD cells, for T cell alloreactivity specifically directed against the injected MSC.

4.4.1. PLX-PAD cells have a comparable capacity as BM-MSC to inhibit the ability of mDC to induce NK cell activation and Th1 priming *in vitro*

Most of our *in vitro* experiments were performed with BM-MSC, while PLX-PAD were used in the clinical trial. Here, we first compared the immunomodulatory capacity of BM-MSC and PLX-PAD cells with regard to DC/NK/T cell cross talk. We measured IFNγ production by NK or T cells after activation by mDC, which have been matured in the presence or absence of BM-MSC or PLX-PAD, respectively.

As shown in Figure 35A, BM-MSC and even more efficiently PLX-PAD inhibited the ability of mDC to induce Th1 priming of allogeneic naïve T cells *in vitro*, as reflected by reduced IFNγ production after five days of co-culture. Moreover, no Th1 priming specific to allogeneic BM-MSC or PLX-PAD cells could be observed in our experiments (Figure 35A). Furthermore, BM-MSC and PLX-PAD exhibited the same inhibitory rate when analysing their influence on the ability of mDC for NK cell activation, represented by their IFNγ production (Figure 35B).

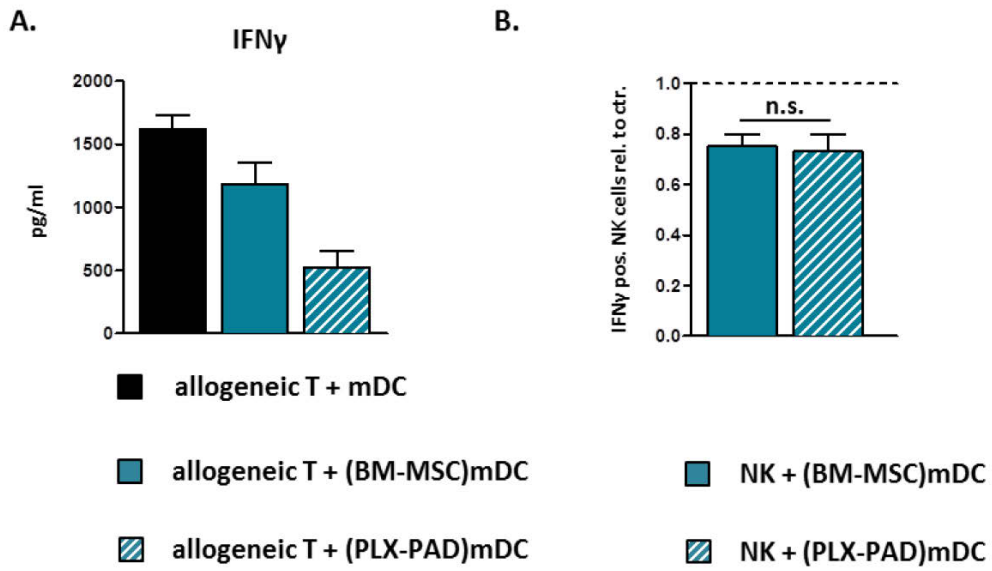

Figure 35: PLX-PAD and BM-MSC have a comparable capacity to inhibit the ability of mDC to activate naïve T and NK cells *in vitro.* (A-B) Mature mDC (black), (BM-MSC)mDC (blue) or (PLX-PAD)mDC (striped blue) were used for activation of naïve T or $CD56^{bright}$ NK cells. IFNγ production was investigated by CBA (A) or flow cytometry (B). **(A)** Naïve T cells were stimulated with the specified allogeneic mDC for five days. Mean IFNγ levels in the culture supernatants +/- SEM are depicted (n=4). **(B)** $CD56^{bright}$ NK cells were co-cultured with mDC as indicated for 24hrs. Percentage of $IFN\gamma^{+}$ NK cells relative to control without MSC (dashed line) is shown as mean +/- SEM.

4.4.2. Allogeneic PLX-PAD do not induce a T cell alloresponse *in vitro*

In order to test if PLX-PAD cells induce a T cell response *in vitro*, we cultured naïve or memory $CD4^+$ T cells with autologous moDC and measured T cell proliferation by CFSE dilution after six days. PLX-PAD were either present during moDC generation or during T cell stimulation. In both cases, PLX-PAD did not induce T cell activation, neither in naïve nor in memory $CD4^+$ T cells (Figure 36).

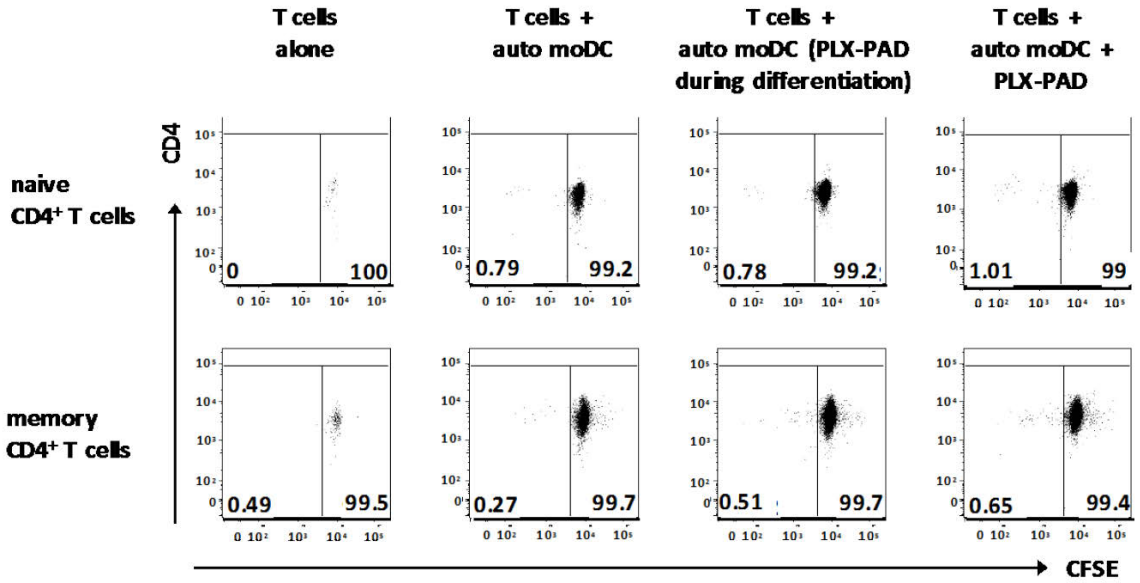

Figure 36: Allogeneic PLX-PAD do not induce an alloresponse of $CD4^+$ T cells *in vitro*. Mature moDC were used to activate autologous (auto) naïve (upper panel) or memory (lower panel) $CD4^+$ T cells for six days. PLX-PAD were either present during differentiation/maturation of moDC or during T cell stimulation. Percentages of proliferated ($CFSE^-$) T cells were determined by flow cytometry. One representative donor out of five is shown.

4.4.3. Allogeneic MHC-unmatched PLX-PAD cells do not induce Th1 priming specific for the MHC mismatch in severe critical limb ischemia (CLI) patients

Samples from CLI patients treated with HLA-unmatched PLX-PAD cells were tested for *in vivo* Th1 priming specific for the respective HLA mismatches between PLX-PAD donor and recipient. PBMC were isolated from patient blood before, three days, one week and four weeks after PLX-PAD injection and were stimulated *ex vivo* by the respective PLX-PAD cells or unrelated third party donor PBMC. The sensitive IFNγ Elispot test was used to measure *in vivo* induced Th1 responses (Figure 37). Only results that exceeded the background plus twofold standard deviation (SD, up to 23 IFNγ spots/$3x10^5$ PBMC) were considered as positive, while only responses of more than 25 IFNγ spots/$3x10^5$

PBMC were considered clinically relevant as previously shown [177]. We detected no or only negligible Th1 priming specific for allogeneic PLX-PAD cells (Figure 37A). Only three patients developed a minimal response towards the donor cells (indicated with open symbols). One of these patients (open triangle) already showed a preexisting low cross-reactivity towards donor PLX-PAD as well as against unrelated third party donor cells at the beginning of the study. This indicates an unspecific "bystander" response, as it can e.g. happen after vaccination. Nevertheless, this preexisting response was not amplified during the clinical trial. Two other patients (open circle and open square) showed a marginal response one or four weeks after PLX-PAD injection respectively, which we could not detect in response to third party cells. However, none of the patients reached a response that exceeded the background or could be considered as clinically relevant. Therefore, we can conclude that PLX-PAD cells did not induce Th1 priming specific for the respective HLA mismatches between PLX-PAD donor and recipient in this clinical trial.

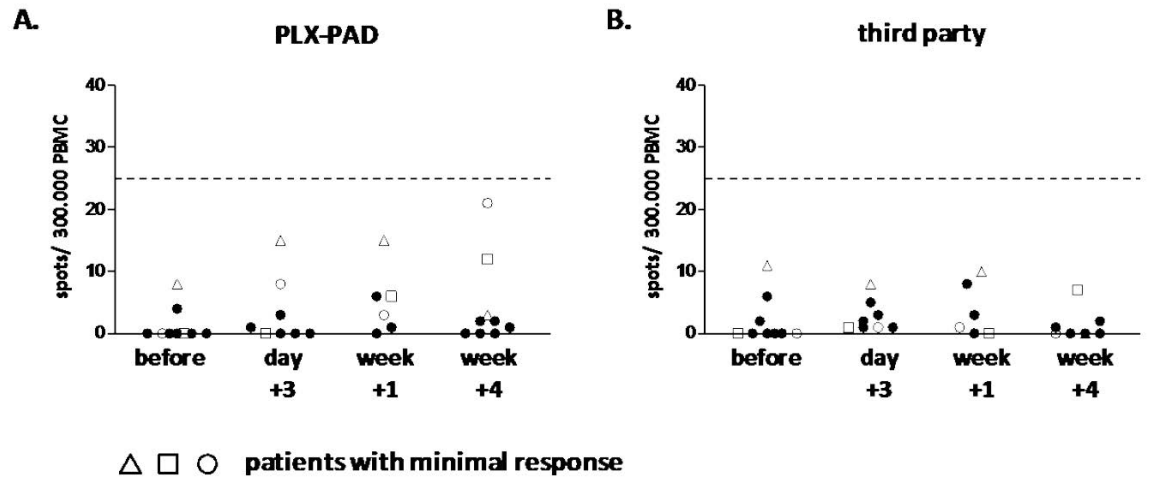

Figure 37: Allogeneic PLX-PAD cells do not induce Th1 priming specific for the MHC mismatch *in vivo*. (A-B) CLI patients received i.m. injections of PLX-PAD cells into the affected limb once. Blood was drawn before, three days, one week and four weeks after treatment. PBMC were stimulated in triplicates at IFNγ Elispot plates by PLX-PAD **(A)** or third-party allogeneic PBMC **(B)** overnight. The number of spots per $3x10^5$ PBMC was corrected for the background in unstimulated controls. A response above 25 spots/$3x10^5$ PBMC has been defined as clinically relevant (dashed line). Open symbols indicate three patients with marginal reactivity towards the mismatch.

4.5. Staining of MSC in human placenta tissue sections

MSC are characterised by a set of surface markers. They express CD73, CD90 and CD105 (≥95%), while they lack expression of CD45, CD34, CD14 or CD11b, and CD19 or CD79α (≤2%). Due to the limited number of biomarkers that can be detected by immunohistochemistry, it was not possible to stain MSC in tissue sections until now. Chipcytometry is a powerful new technique that combines the advantages of flow cytometry and microscopy and allows investigation of cell suspensions or tissue cryosections [178]. Hence, chipcytometry offers the possibility to stain human MSC in tissue sections for the first time.

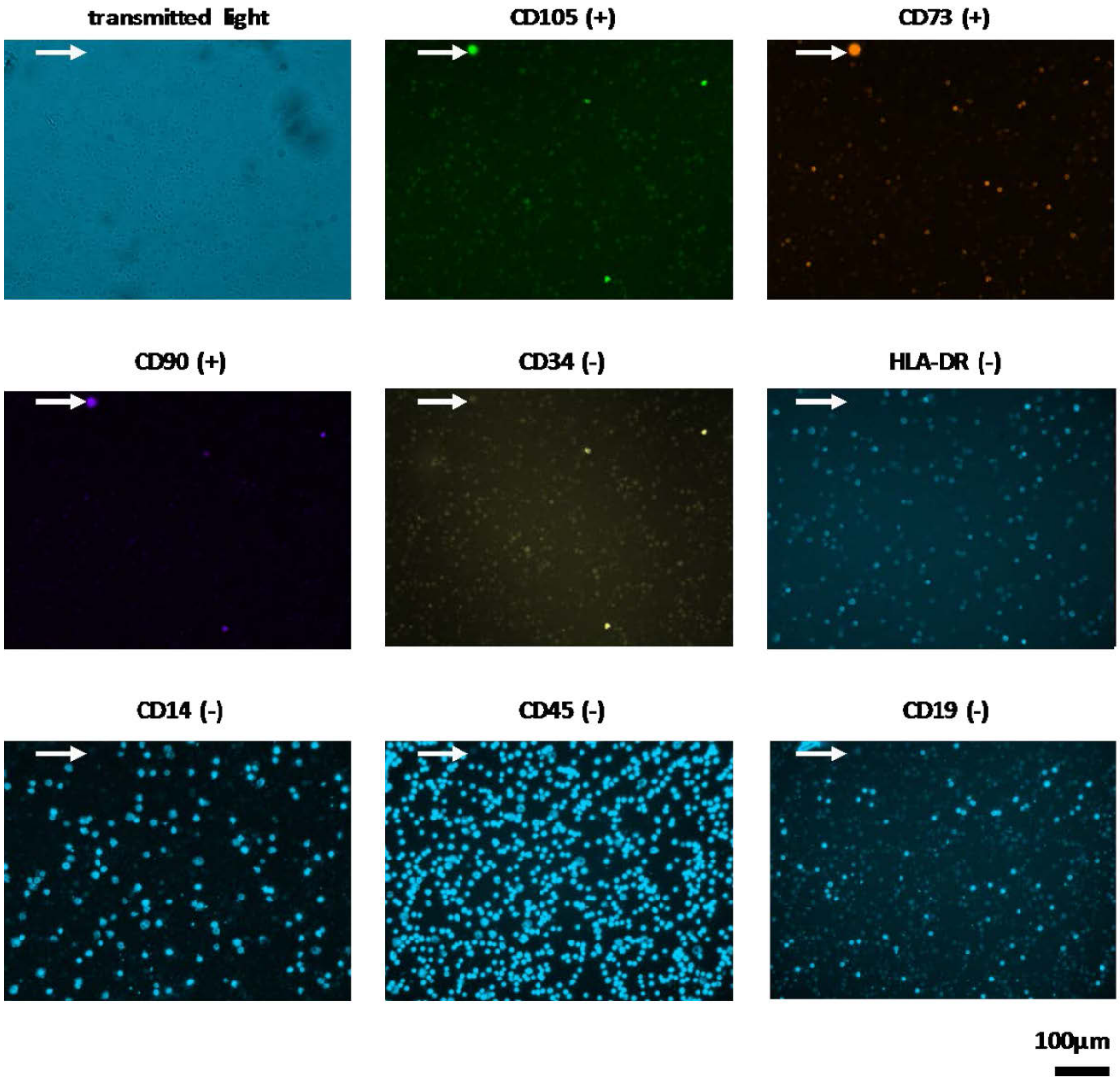

Figure 38: Detection of BM-MSC in PBMC dilutions. PBMC were spiked with human BM-MSC for an approximate dilution of 50,000:1. Cells were loaded onto Zellsafe_C chips. Samples were stained for CD73, CD90 and CD105 (positive markers) as well as CD14, CD19, CD34, CD45 and HLA-DR (negative markers). One MSC was detected among $1x10^5$ scanned cells (white arrow).

We spiked PBMC with human BM-MSC for an approximate dilution of 50,000 PBMC to one BM-MSC. Chips were stained for CD73, CD90 and CD105 (positive markers) as well as CD14, CD19, CD34, CD45 and HLA-DR (negative markers). $1x10^5$cells were scanned and one BM-MSC could be detected (Figure 38).

MSC can be isolated from many different tissues, such as bone marrow, adipose tissue, placenta or umbilical cord. Here, we analysed cryosections from human placenta for $CD73^+$ $CD90^+$ $CD105^+$ CD45- CD34- CD14- CD19- cells. Figure 39 shows one representative staining of a placenta section that arose from a full term pregnancy. In the position presented in Figure 39, three MSC were detected. In total, six MSC were found in this slide. Moreover, we also found three MSC in a sample from a placenta deriving from an abortion (at the most 12^{th} week of pregnancy, data not shown), showing that MSC can be detected in placentas deriving from late as well as from early pregnancies.

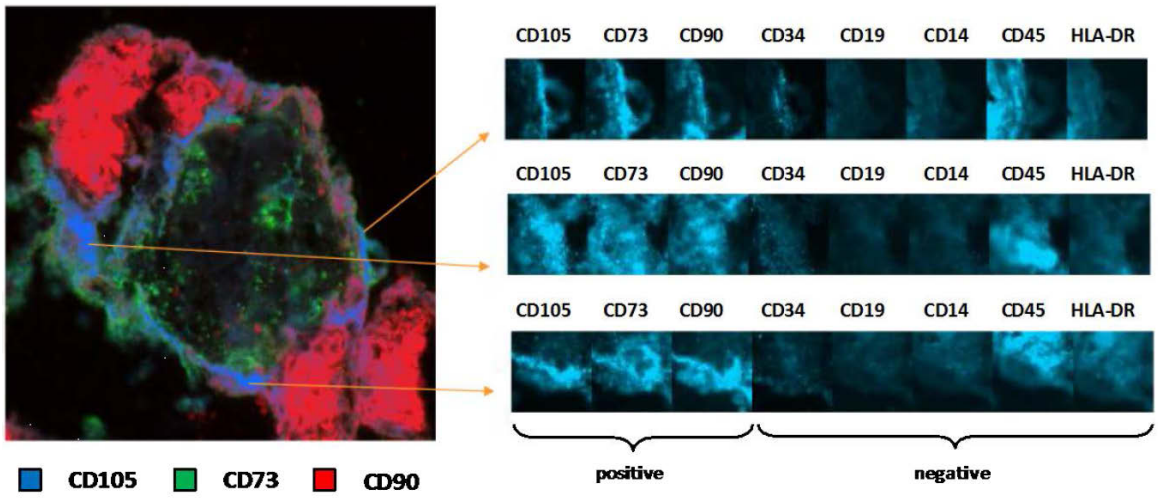

Figure 39: Detection of MSC in human placenta tissue sections using chip cytometry. 5µm cryosections were prepared from a full term pregnancy placenta. Sections were transferred on Zellsafe_T chips and fixed. Samples were stained for CD73, CD90 and CD105 (positive markers) as well as CD14, CD19, CD34, CD45 and HLA-DR (negative markers). In total, six MSC were found in the section. One representative donor out of two is shown.

5. DISCUSSION

This doctoral thesis aimed to gain a better understanding of MSC's impact on the crosstalk between human DC, NK cells and T cells.

Firstly, we could show that BM-MSC induced a phenotype for tolerogenic DC when present during maturation of *in vivo* generated $CD1c^+$ mDC. In contrast, they inhibited differentiation but not maturation of *in vitro* generated moDC and had only minor effects on maturation of *in vivo* generated pDC.

Secondly, we gained a better understanding of how BM-MSC influence the crosstalk between DC, NK and T cells by interfering with mDC maturation. BM-MSC-licensed mDC displayed a lower ability to activate NK cells, most likely due to a reduced IL-12 secretion. IL-10 was the key player regulating the altered cytokine production. BM-MSC-licensed mDC were also less efficient in inducing priming of naïve T cells into Th1 effector cells.

Thirdly, we could confirm the low alloimmunogenicity of MHC-unmatched MSC-like cells (PLX-PAD) after *in vivo* application in CLI patients. None of the patients developed a significant memory T cell response specific to the allogeneic PLX-PAD cells. Since PLX-PAD inhibited mDC's capacity for NK and T cell activation to a comparable extent as BM-MSC, the presented *in vitro* data could explain these *in vivo* effects.

Finally, we detected for the first time MSC *in situ* on human placenta tissue cryosections by staining for $CD73^+$ $CD90^+$ $CD105^+$ $CD45^-$ $CD34^-$ $CD14^-$ $CD19^-$ cells using the novel chipcytometry technique.

5.1. BM-MSC inhibit *in vitro* differentiation of human moDC, whereas they do not affect the maturation process

MSC were previously shown to interfere with *in vitro* differentiation of human monocytes and $CD34^+$ precursors into DC, resulting in a reduced expression of maturation markers and a decreased ability for T cell stimulation [135,137,139,145]. It is less well understood whether MSC also interfere with the maturation of already differentiated immature DC [137,139].

DC maturation is characterized by three main properties: CCR7-dependent migration towards lymph nodes, secretion of pro-inflammatory cytokines/chemokines and antigen presentation in combination with supply of costimulatory signals for T cells. In accordance with other publications [135,179], we observed a significantly higher remaining percentage of $CD14^+$ cells when BM-MSC have been present during the differentiation process, showing that monocytes mainly remained undifferentiated or insufficiently differentiated. These moDC generated in the presence of BM-MSC also acquired lower expression of CD40, CD83 and CD86 than control moDC, whereas CD80 as well as HLA-DR expression were not altered. Moreover, CCR7-dependent migration and secretion of IL-12p70, IL-1β and TNFα were decreased. Finally, we also observed a reduced ability to induce the proliferation of naïve and memory $CD4^+$ T cells, while IFNγ release by both T cell subpopulations was affected in different ways. Naïve T helper cells secreted less IFNγ after priming and restimulation by moDC that have been generated in the presence of BM-MSC, while memory $CD4^+$ T cells were still able to produce IFNγ. This shows that MSC suppress the ability of moDC to induce lineage decision and proliferation of naïve T cells towards the Th1 compartment. Since memory T helper cells have already been primed *in vivo*, they do not need professional DC to get activated. IFNγ release by memory T cells might only be induced through TCR signalling, while naïve T cells need additional DC-derived IL-12 [108,188]. On the contrary, T cell proliferation of both the naïve and memory compartment is also induced by cytokines [189,190] and therefore was reduced in both experimental settings. In summary, Th1 priming of naïve T cells is decreased when activated by moDC that have been generated in the presence of BM-MSC, while memory T cells do not need professional APC to start their effector program. However, the total number of IFNγ-producing memory T cells should be decreased since they proliferated less.

Until now, there are only few publications that dissect the impact of MSC on differentiation and maturation of DC and the presented results are contradictory. They cover the whole spectrum of effects: from no effect at all over moderate or strong inhibition to enhancement of maturation [137,139,141,191]. In our study, in contrast to clear effects on moDC differentiation, BM-MSC did not affect expression of maturation markers and costimulatory molecules when they have been present only during LPS-

induced *in vitro* maturation of already differentiated immature moDC. This clearly shows that most previously published data about inhibited expression of maturation markers in the presence of MSC need to be led back to the disturbed DC differentiation and not to inhibition of DC maturation itself. It has to be concluded that MSC block the differentiation of monocytes and $CD34^+$ precursors into DC. As a consequence, these incomplete DC do not upregulate expression of costimulatory molecules and secretion of pro-inflammatory cytokines, such as IL-12 being a typical cytokine secreted by DC but not by monocytes. The final outcome of these effects is a reduced potential for T cell activation.

Generally, the interpretation of published data on this topic is complicated by the fact that different DC sources or times of MSC administration have been used in different studies. Especially, monocytes, the main source for DC generation *in vitro*, do not give rise to conventional mDC *in vitro* and *in vivo*, since they rather differentiate into inflammatory DC [63,67,72]. To my knowledge, Aggarwal et al. published the only known data using another DC source, namely human $CD1c^+$ mDC and $CD304^+$ pDC [147].

5.2. BM-MSC show no clear effect on *in vitro* maturation of human blood-derived pDC

So far, only one study used *ex vivo* isolated human pDC, which were matured by IL-3 in the presence or absence of MSC [147]. The authors could show that MSC-licensed pDC secreted significantly higher amounts of IL-10, the only cytokine, which has been measured. Yet, IL-10 is not the key cytokine secreted by pDC and other cytokines or properties for maturation were not investigated.

Here, we demonstrate for the first time that BM-MSC do not have a strong influence on *in vitro* maturation of blood-derived $CD304^+$ pDC. In general, pDC are known to have little, if any, capability for antigen presentation and are rather considered as immunomodulatory cells by directing the immune response towards an antiviral response [192]. Here, expression of the only clearly up-regulated markers CD40 and HLA-DR was not affected by BM-MSC. Although the percentage of CCR7 expressing cells as well as the level of CCR7 per cell remained unchanged in the presence of BM-MSC, the number of migrating pDC was still significantly reduced. Regarding cytokine

secretion of pDC, we obtained inconsistent results. IFNα and TNFα are the two most important cytokines secreted by pDC. While IFNα secretion was increased in pDC/BM-MSC co-cultures, TNFα production was reduced. In addition, we detected higher levels of the chemokines IP-10, MCP1 and IL-8 as well as of the pro-angiogenic growth factor VEGF.

Directed cell migration not only depends on the presence of the chemokine receptor and its specific ligand(s), but also on other factors such as signalling downstream of the chemokine receptor, cytoskeletal rearrangement (mainly of actin and myosin) or expression of adhesion molecules (e.g. vascular cell adhesion molecule [VCAM] 1, ICAM1) [193,194]. Regarding the last point, we did not detect an influence of BM-MSC on the secretion of soluble VCAM. This is at least an indication that the reduced *in vitro* migration of BM-MSC-licensed pDC is not due to differences in expression of adhesion molecules. Binding of CCL19 or CCL21 to CCR7 leads to activation of two different signalling cascades, one inducing the chemotactic response and the other one enhancing the migratory speed [195]. On the one hand, CCR7 signalling induces activation of G_i, a subfamiliy of G proteins, leading to activation of p38, extracellular-signal-regulated kinases 1/2 (ERK 1/2) and c-Jun N-terminal kinases (JNK), finally regulating chemotaxis. On the other hand, CCR7 activation induces signalling via Rho GTPases. The Rho/Pyk2/Cofilin pathway is known to control actin organization and therefore regulates the migratory speed. Interestingly, it has been shown previously in bone marrow-derived mouse DC that *in vitro* phosphorylation of ERK1/2 and p38 upon LPS stimulation of TLR4 is reduced in the presence of MSC [132]. Both signalling cascades are also involved in TLR activation of pDC [196]. If MSC inhibit phosphorylation of ERK1/2 and p38 in human pDC and whether they do so not only upon TLR stimulation but also upon CCR7 stimulation could be further investigated for instance by Western Blot or FACS.

pDC developed an elegant mechanism to uncouple the activation of innate and adaptive immune responses, although both pathways are activated by TLR7/9 signalling. Different TLR9 ligands are directed either in the early (e.g. CpG-A) or late (e.g. CpG-B) endosomes [172] resulting in the activation of different signalling cascades. Activation of the transcription factor interferon regulatory factor (IRF) 5 via early endosomes leads to production of pro-inflammatory cytokines, such as TNFα and IL-6, thereby activating the

adaptive immune system [197]. In contrast, IRF7 activation via late endosomes induces secretion of type 1 interferons, mainly IFNα, resulting in an innate and anti-viral immune response [44,172]. We observed higher release of IFNα, but lower TNFα levels in our pDC/BM-MSC co-cultures compared to mature pDC alone. One explanation might be that MSC influence the different endosomal and signalling pathways in a different way. In the future, we could analyse activation of IRF5 and IRF7 in pDC upon co-culture with MSC. In contrast to the lower TNFα production, the level of IL-6 in pDC/BM-MSC co-cultures was increased. According to the literature [172,198,199], we observed IL-6 release by both cell types when they were cultured alone. BM-MSC even secreted high IL-6 levels in the absence of TLR ligands. Thereby, we cannot clearly identify the source of IL-6 in the co-culture system. Some of the immunomodulatory effects of MSC previously have been linked to MSC-derived IL-6, especially suppression of DC differentiation [199,200,201]. However, it is generally complicated to distinguish the source of soluble factors when analysing the culture supernatant of co-culture systems. It was not possible to separate BM-MSC and DC after a short incubation period and to analyse the secretion profile of both cell types afterwards, since most of the soluble factors relevant in our setting are released within a short time frame. Intracellular staining of these factors is rather complicated and does not work very well. Another important issue is that intracellular staining requires addition of secretion inhibitors such as Brefeldin A or Monensin. These block secretion of all soluble factors in all cells present in the culture system and thereby might interfere with the immunomodulatory effects of MSC, too. One could try to perform intracellular staining without secretion inhibitors, but most likely the intracellular concentration of the factors of interest would be below the detection limit. Another possibility would be to analyse RNA and protein expression within individual cells, either by single cell PCR or by flow cytometry using the PrimeFlow RNA assay.

The increased levels of the chemokines IP-10, MCP1 and IL-8 in pDC/BM-MSC co-cultures would attract a range of different immune cells. IP-10 is an important pro-inflammatory chemokine for the initiation of immune responses since it attracts a variety of innate as well adaptive immune cells to inflamed tissues, mainly monocytes, macrophages, NK cells, DC and activated Th1 cells [202,203]. MCP1 is another pro-

inflammatory chemokine recruiting monocytes, DC and T cells to sites of inflammation [204]. Interestingly, it causes decreased IL-12 production and increased Th2 priming. IL-8 attracts primarily neutrophils but also other granulocytes to sites of infection and activates their phagocytic capacity [205]. Additionally, it can promote angiogenesis by mimicking the function of VEGF [206]. As shown in Table 11 and by other authors, IP-10, MCP1 and IL-8 could be secreted by pDC as well as by BM-MSC [207,208,209]. Most probably, the increased levels of these chemokines in the co-culture are a mixture secreted by both cell types. In an *in vivo* situation, the broad panel of immune cells attracted by these chemokines would of course provide a good opportunity for MSC to modulate their functionality, even indirectly by modifying pDC functionality. It is also interesting that we not only observed high secretion of IL-8 in pDC/BM-MSC co-cultures but also of VEGF, being one of the most important pro-angiogenic factors. However, we even detected a higher VEGF level when BM-MSC were cultured alone, confirming previous data showing high VEGF release by MSC [29]. Moreover, it has been reported that VEGF production by MSC is enhanced in the presence of IL-8 [210]. Our observations support the hypothesis that MSC are a good option for the treatment of angiogenic disorders, for instance CLI.

5.3. BM-MSC suppress *in vitro* maturation of human mDC

The only known data about the interaction between human MSC and $CD1c^+$ mDC were published by Aggarwal and colleagues [147]. Unfortunately, they merely showed reduced TNFα production in the presence of MSC, neglecting many other cytokines and characteristics of DC maturation. Our work represents the first known large study showing suppressed *in vitro* maturation of human blood-derived $CD1c^+$ mDC by MSC.

We could show that BM-MSC affected the three hallmarks of mDC maturation to different extents. They did not prevent upregulation of molecules for antigen-presentation and costimulation of naïve T cells by mDC. Expression of HLA-DR, CD80 and CD86 on BM-MSC-licensed mDC was even marginally increased. Spaggiari et al. reported similar data for maturation of *in vitro* generated moDC [137]. By contrast, the levels of CD83 and CD40 were diminished in the presence of BM-MSC. The exact function of CD83, which is used as one of the most important markers for fully mature DC, is not

completely understood yet. Nevertheless, there is some evidence that CD83 plays a role for stimulation of allogeneic T cells [211]. Beside a relatively strong suppression of CD83 acquisition, we observed a moderate reduction in CD40 expression. In brief, the biological significance of both modifications by MSC remains questionable. This correlates with our finding that BM-MSC do not interfere with the acquisition of maturation markers by moDC and pDC, which were matured by TLR ligands in the presence of BM-MSC. In total, MSC do not have a strong impact on the acquisition of costimulatory molecules and maturation markers when they encounter an immature DC.

Until now, investigating migration of MSC-licensed DC has not been of deep interest in the field. Murine MSC were shown to suppress CCR7 acquisition by bone marrow-derived murine DC *in vitro* and *in vivo* [132,140,141], while it has not been questioned how CCR7 expression and migration are affected in the human system. We show for the first time that BM-MSC-licensed mDC acquire less CCR7 when matured by low dose LPS, resulting in a diminished migration towards the receptor's ligand CCL21. *In vivo*, this would lead to reduced migration of DC from sites of inflammation into draining lymph nodes, finally resulting in a decreased activation of NK and T cells. However, the inhibitory effect of BM-MSC could be overcome by high dose TLR stimulation using a combination of LPS and R848, which is needed to efficiently induce cytokine production by mDC *in vitro* [173]. Thus the outcome *in vivo* might depend on the level and the composition of TLR ligands encountered by the DC as well as on its actual differentiation status.

The most prominent change in BM-MSC-licensed mDC was their altered cytokine secretion profile. They produced significantly decreased levels of IL-12p70 and IL-1β and higher levels of IL-10 and IL-1Ra. In the mDC/BM-MSC co-culture, the ratios of pro-inflammatory IL-12 and IL-1β to anti-inflammatory IL-10 and IL-1Ra respectively were strongly shifted towards the anti-inflammatory side, representing a cytokine profile typical for tolerogenic DC. Blocking experiments revealed a central role for IL-10 causing these changes. IL-1β and IL-1Ra regulated IL-12 production, while IL-1Ra also counteracted IL-1β production. IL-10 was the only cytokine enhancing the level of IL-1Ra and at the same time inhibiting IL-12 and IL-1β production. Interestingly, IL-10 is one

possibility to generate tolerogenic DC *in vitro* [92]. Furthermore, IL-10 has been proposed as a mode of action by which MSC suppress DC generation. In line with our findings using neutralizing antibodies, IL-10 has been shown earlier to inhibit the secretion of IL-12 or IL-1β, whereas it induces the production of their natural antagonists, for example IL-1Ra (see paragraph 4.3.5.1) [212,213]. Thus the high levels of IL-1Ra in mDC/BM-MSC co-cultures could either be a direct effect of BM-MSC on IL-1Ra production or could be mediated via increased IL-10 levels. IL-1Ra in turn played at least a partial role for the regulation of IL-12 and IL-1β levels in mDC/BM-MSC co-cultures. By blocking its signalling, IL-1Ra is the natural antagonist for IL-1β [214]. Unfortunately, we could not specifically measure biologically active IL-1Ra in the presence of its blocking antibody (most probably due to different epitopes recognized by the blocking and neutralizing antibodies), while biologically active IL-10 and IL-6 were absent under neutralizing conditions. Yet, we cannot distinguish whether IL-10 and IL-6 release by mDC is indeed reduced (due to aborted positive feedback loops) or if only the biologically active level of these cytokines is reduced under neutralizing conditions. For future experiments, it would be interesting to support our assumption that IL-10 is the key player by investigating IL-10 signalling in mDC. The starting point would be to analyse phosphorylation of STAT3, which is the central transcription factor activated in the signalling cascade [215], by Western Blot, flow cytometry, CBA or Elisa.

So far, the proposed mechanisms by which MSC inhibit the functionality of DC vary and are influenced by the experimental setting, the species and the context in which MSC encounter DC or the respective precursors. Mainly soluble factors were reported with PGE2 being the most considered mediator for suppressed DC differentiation [137,144]. However, we were not able to reproduce these data (data not shown). Other studies proposed a role for IL-10, IL-6 or M-CSF, but only when MSC have been present during early DC differentiation [39,135,139]. Here, we show for the first time that also during short-term maturation of *in vivo* generated mDC, MSC can drastically influence the cytokine production by DC resulting in an anti-inflammatory profile typical for tolerogenic DC. As a consequence, the activation of T and NK cells might be inhibited. For example, DC-derived IL-10 drives the polarization of naïve T cells into IL-10-secreting Treg [216]. Moreover, IL-10 influences DC and other immune cells in their immediate

vicinity (e.g. Th1 cells, NK cells and macrophages), thereby amplifying the tolerogenic circuit in an auto- and paracrine manner [182,212]. Since IL-1β is important for IL-12-mediated IFNγ secretion by NK cells, its reduced production in the presence of MCS might negatively regulate NK cell activation. Finally, IL-12 is important for type 1 immune responses by NK and T cells [217]. We could not definitely determine the source of IL-10 and IL-1Ra in our mDC/BM-MSC co-cultures due to different reasons discussed earlier (see section 5.2). In accordance to other publications [145,147], we did not detect IL-10 secretion when BM-MSC were cultured alone. Nevertheless, other authors reported that MSC are indeed able to produce IL-10 [218,219,220]. Murine MSC were shown before to secrete IL-1Ra, which played a central role for their anti-inflammatory and anti-fibrotic effects in a mouse model of lung injury [221]. As in the case of IL-10, our BM-MSC alone did not secrete IL-1Ra, whereas mDC alone produced IL-1Ra and its release was strongly increased in mDC/BM-MSC co-cultures. At the same time, the level of IL-1β was strongly diminished in the presence of BM-MSC.

Beside the considerable changes in cytokine production by mDC in the presence of BM-MSC, we also observed a strong influence on secretion of chemokines and growth factors. As in the case of pDC, we detected significantly higher levels of the chemokines IP-10 and MCP1 and a tendency for increased IL-8 release. Nevertheless, the origin of these chemokines in our culture cannot be determined with clarity. MSC were shown before to produce all of the three chemokines [209,222,223]. $CD1c^+$ mDC release high amounts of IL-8 in response to many TLR agonists, while IP-10 and MCP-1 are only secreted upon specific TLR stimulation [74,181]. Most probably, all factors could be secreted by both cell types in the co-culture. These chemokines mainly recruit innate immune cells, such as neutrophils and monocytes, but also activated T cells, to sites of infection or MSC injection [224]. This could enable MSC to modulate their functionality directly or indirectly by modifying DC and to influence the course of the (allo)immune response.

VEGF represents a critical factor in physiological and pathological regulation of angiogenesis and is for instance involved in formation of new blood vessels from preexisting vessels and in wound healing [225]. MSC were shown before to secrete high amounts of VEGF [29,222]. GM-CSF is a white blood cell growth factor, inducing

differentiation of granulocytes and monocytes from precursors [226]. Though, GM-CSF also functions as an immunomodulatory cytokine, capable to affect T cell activation [227]. G-CSF mainly acts on neutrophils by stimulating their proliferation and differentiation in the bone marrow and controlling their release into the bloodstream [228]. Moreover, it increases survival and functionality of mature neutrophils [229]. Although, secretion of G-CSF and GM-CSF by MSC from other tissue sources has been reported [223,230,231], we did not detect these factors when BM-MSC were cultured alone. In contrast, TLR ligand stimulated mDC alone secreted high amounts of both growth factors and their levels were highly enhanced in mDC/BM-MSC co-cultures. In total, the increased release of chemokines, growth factors and factors controlling angiogenesis in the mDC/BM-MSC co-culture indicates a high capacity for wound healing *in vivo*, especially being useful in the context of chronic ischemic injuries, such as CLI [230].

5.4. Impact of BM-MSC on the crosstalk between mDC, NK cells and T cells *in vitro*

So far, only the isolated impact of MSC on either DC, NK or T cells has been investigated. However, the crosstalk between DC, NK and T cells is essential for the initiation of immune responses, especially for Th1 responses. Their interaction mainly takes place in secondary lymphoid organs or inflamed peripheral tissues [94,115]. Due to different reasons, we decided to use mDC as APC for our investigations on this important network. Blood-derived mDC are classical DC and therefore the most important APC for induction of immune responses. moDC have to be generated *in vitro*, leading to more instability of the experimental setting. Moreover, our results regarding the pDC were (partially) contradictory and not as straightforward as for mDC. Using pDC would require more investigations as discussed in section 5.2. In sum, mDC represented the best option as APC to analyse the influence of BM-MSC on the crosstalk between DC, NK and T cells. In brief, *in vivo* activation of naïve T cells is controlled by several checkpoints, which involve DC and NK cells (see section 1.2.5) [116,117,118,119]:

i. DC migration to draining lymph nodes
ii. Chemokine release by DC leading to recruitment of NK, T and B cells
iii. Secretion of pro-inflammatory cytokines by DC

iv. NK cell activation including IFNγ secretion, being essential for Th1 priming

v. Antigen presentation and costimulation by DC

Regarding the first point, BM-MSC suppressed the ability of mDC to migrate towards secondary lymphoid organs, leading to reduced opportunities for priming of lymph node resident NK and naïve T cells. Furthermore, we observed a higher level of pro-inflammatory chemokines IP-10 and MCP1, recruiting mainly monocytes, macrophages, DC, NK cells and activated Th1 cells to inflamed peripheral tissues. From both results we can conclude that BM-MSC-licensed mDC rather remain in the tissue where other effector cells are recruited to.

The shift of cytokine production from a pro-inflammatory to an anti-inflammatory milieu had strong effects on the ability of these DC to activate NK cells in terms of proliferation, cytotoxic ability as well as IFNγ production. BM-MSC indirectly diminished the amount of early NK cell-derived IFNγ by interfering with mDC maturation. The lower IL-12 production by BM-MSC-licensed mDC turned out to be the reason for their reduced capacity to induce IFNγ production in NK cells. We could also show that the increased IL-10 level in mDC/BM-MSC co-cultures was the reason for the diminished ability of mDC for NK cell activation. Our data indicate that BM-MSC favour the generation of tolerogenic mDC in an IL-10-dependant manner [92].

The diminished IFNγ production by NK cells is a very important finding regarding the interplay between DC, NK and T cells (Figure 4). NK cell-derived IFNγ is known to directly and indirectly promote and sustain Th1 immune responses. Cytokines secreted by DC (among them mainly IL-12) are known to efficiently induce proliferation, enhanced cytolytic ability and IFNγ production in NK cells [123,124,125]. In turn, IFNγ secreted by NK cells amplifies maturation and IL-12 release of DC, leading to improved Th1 priming [103,120,126,127]. Moreover, NK-cell derived IFNγ directly increases Th1 lineage commitment of naïve T cells by enhancing their IFNγ production and the expression of IL-12 receptor, which facilitates binding to DC-derived IL-12 [128]. Hence, the synergy between IFNγ and IL-12 leads to full Th1 differentiation [106]. This thesis clearly shows that both, IL-12 production by mDC and IFNγ release by NK cells, are strongly reduced when MSC interfere with mDC maturation. Moreover, the reduced IFNγ production by

NK cells could be traced back to the decreased IL-12 and increased IL-10 production of BM-MSC-licensed mDC. In addition to their effects on the crosstalk between DC, NK and T cells via reduced NK cell activation, BM-MSC-licensed mDC were also less efficient to directly induce differentiation of allo-reactive naïve T cells into IFNγ-producing Th1 memory cells. This was most probably also due to their diminished IL-12 release.

Nevertheless, BM-MSC did not affect expression of HLA-DR and costimulatory molecules CD80 and CD86, suggesting that antigen-presentation to T cells remains conserved. However, BM-MSC-licensed mDC expressed less CD83 and CD40, two other characteristic markers for DC maturation. Interestingly, it has been shown that downregulation of CD83 in moDC strongly reduced their capacity to stimulate allogeneic T cells in terms of proliferation and cytokine production [211,232], indicating that CD83 acts as an essential enhancer during the activation of allo-reactive T cells. Furthermore, engagement of CD40 on the DC surface to CD40L on activated T cells stimulates their cytokine production (e.g. IL-12), enhances the expression of costimulatory molecules on their cell surface and facilitates cross-presentation [233]. Overall, CD40 signalling is needed for full DC maturation and to achieve all of the necessary characteristics of an effective triggering of T cell activation and differentiation [234]. Therefore, the reduced levels of CD83 and CD40 might contribute to the diminished capacity of BM-MSC-licensed mDC for Th1 priming of allogeneic T cells - despite their preserved antigen presentation.

In summary, by generating tolerogenic mDC, MSC affect all of the five checkpoints for Th1 priming (Figure 40), although each one to a different extent. The sum of all effects could be the explanation for the described inhibition of Th1-driven immune-pathological processes by MSC, for instance in the case of GvHD, and their escape from immune recognition *in vivo* [36].

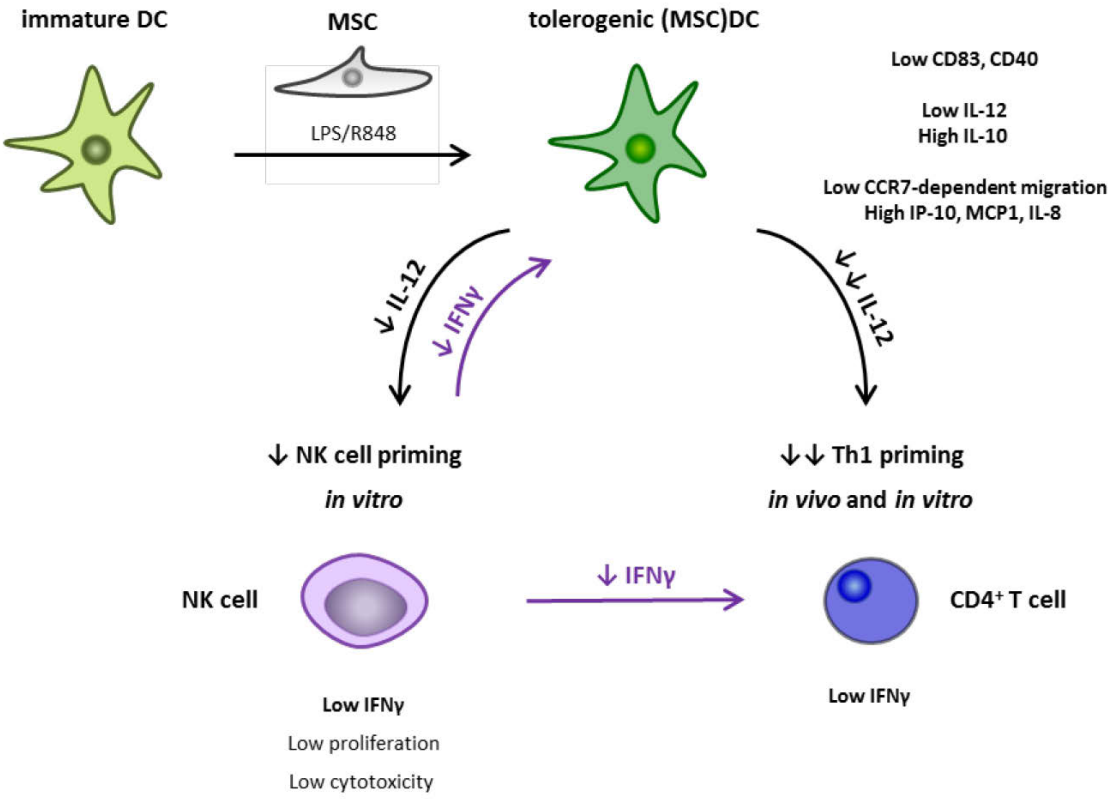

Figure 40: By generating tolerogenic DC, MSC interfere with the crosstalk between DC, NK cells and T cells. After antigen encounter in the periphery, DC start to migrate in a CCR7-dependent manner towards lymph nodes and produce pro-inflammatory cytokines, e.g. IL-12. DC-derived IL-12 induces Th1 priming of naïve T cells and activation of NK cells. IFNγ produced by activated NK cells enhances IL-12 release by DC and directly acts on T cells. Both pathways lead to increased Th1 priming. mDC matured in the presence of MSC display a phenotype of tolerogenic DC with IL-10 being the key mediator for these changes. MSC-licensed mDC produce less IL-12, resulting in a lower ability for Th1 priming of allogeneic naïve T cells. Moreover, they display a decreased potential for the activation of autologous NK cells in terms of proliferation, cytotoxicity and IFNγ production, with the latter being essential for priming of Th1 responses, directly by acting on T cells or indirectly by modifying DC cytokine production.

5.5. MSC-like PLX-PAD cells do not induce Th1 responses *in vivo*

So far, immunological data from CLI clinical trials using human allogeneic MSC are completely missing. We analysed samples from patients that received allogeneic MSC-like PLX-PAD cells for *in vivo* developed memory T cell responses specific for the cell product. Our data demonstrate for the first time that allogeneic off-the-shelf produced HLA-unmatched PLX-PAD cells did not trigger a specific T cell alloreactivity in CLI patients. None of the patients developed a response that exceeded the background or has been defined as clinically relevant [177]. To my knowledge, so far only one study investigated immunological parameters in baboons that have been treated with MSC.

The authors reported reduced T cell responses specific to MSC whereas the overall T cell response to Concanavalin A remained unaffected [235].

Our *in vitro* data show that MSC shift mDC maturation towards a tolerogenic phenotype combined with a reduced migratory capacity towards secondary lymphoid organs. This could lead to a diminished ability of DC for priming of lymph node NK and naïve T cells *in vivo*, thereby explaining the observed *in vivo* effects. Importantly, PLX-PAD cells showed the same or in some experiments even better suppression of mDC's ability to activate NK and T cells *in vitro*. Nonetheless, inhibition of Th1 priming by PLX-PAD cells in patients also could have been directed by direct suppression of T cells. To prove our hypothesis that MSC inhibit the crosstalk between DC, NK and T cells *in vivo* by the mechanisms we identified *in vitro*, further *in vivo* data from patient samples and/or preclinical mouse models are needed. One could take biopsies from the sites of MSC injection in order to analyse markers used in our *in vitro* experiments. It would be interesting to stain for DC and NK cells in the tissue to show their interaction at the site of MSC application. Especially tissue DC could be phenotypically characterized for their expression of surface markers CD83, CD40 and CCR7. Our recently developed technique for staining of MSC in tissue sections(see paragraph 5.6) could be of interest for analysing the fate of MSC injected into patients and the surrounding inflammatory reaction at biopsy material. One could clarify the hypothesized interactions between MSC and immune cells. Most probably, APC are the first cells interacting with the injected MSC. Furthermore, it would be interesting to measure key cytokines such as IL-12, IL-10 and IFNγ in patient blood. In this trial some blood cytokine concentrations were investigated, among them IL-10, which remained, however, unaltered immediately after PLX-PAD application (data not shown). Another possibility would be to measure interesting cytokines in biopsies by digesting the tissue and analysing their concentrations, for instance by using the sensitive Mesoscale multiplex method. However, more data from additional trials for clinical correlation of our *in vitro* findings would be desirable and valuable. Investigating the interaction between MSC and immune cells in humans *in vivo* represents a limitation, since it does not offer the same facilities as animal studies. The clinical data presented here were generated in a phase I/IIa clinical trial with a limited number of patients and the primary goal was to

evaluate safety, determine safe dosage ranges and begin to identify side effects of PLX-PAD cells. Due to this reason, we had only limited space for additional research on immunological questions and were restricted in the amount of patient material that could be used for such purposes. From this trial, we learned a lot about the immunogenicity of MSC and the underlying mechanisms, creating a solid basis for future investigations. Therefore, we were able to implement more biomarkers (e.g. complex flow cytometric phenotyping, gene expression profiling, monocytic function, soluble adhesion molecules etc.) in follow-up studies to better correlate the *in vitro* findings to clinical data. In the next study, one should also analyse functionality of blood-derived mDC and NK cells, especially for their ability to release cytokines.

5.6. MSC can be detected in human placenta cryosections as $CD73^+$ $CD90^+$ $CD105^+$ $CD45^-$ $CD34^-$ $CD14^-$ $CD19^-$ cells

To date, it was not possible to detect MSC in tissue samples since a multiparameter set of CD markers is needed for their identification. Therefore, it is not known if the same set of markers that was established on cultured MSC can be used to detect MSC *in vivo*. Here, we show for the first time that $CD73^+$ $CD90^+$ $CD105^+$ $CD45^-$ $CD34^-$ $CD14^-$ $CD19^-$ MSC can be detected in human placenta tissue sections using chipcytometry, a new technique by which up to 30 biomarkers can be stained at the same time from the same slide. We also plan to establish these staining for adipose tissue and bone marrow, which are the other two most important tissue sources for MSC isolation.

Additionally, there exist a number of candidate MSC surface markers, among them CD29, CD44, Stro-1 or stage-specific embryonic antigen-4 (SSEA-4), which could be analysed in tissue samples in more detail in the future by using this method [236].

Our finding is not only remarkable to generally clarify that MSC carry the same set of biomarkers *in vitro* and *in vivo*, but also allows interesting applications such as analysis of biopsies from MSC-treated patients. As mentioned above (see passage 5.5), it would be interesting to stain for immune cells in the tissue to study which cells are recruited to the injection site and to investigate their interaction with the injected MSC. However, it is rather complicated to distinguish between autologous and allogeneic MSC. One possibility would be to stain for different HLA subtypes, for instance for HLA-$A2^+$ MSC in

an HLA-A2$^-$ patient. Moreover, it is possible to distinguish male and female cells via the sex-determining region Y (SRY) protein. SRY is a transcriptional regulator that controls a genetic switch in male development [237].

Moreover, our technique allows studying the role of MSC in bone marrow niche formation and haematopoiesis in more detail *in vivo* [238]. It has been demonstrated in mice that Nestin-expressing cells in the bone marrow are MSC [239]. Together with HSC, these nestin$^+$ MSC formed a unique bone marrow niche [239]. Nestin was originally found in neuroepithelial stem cells and expressed in the early stages of development [240]. As type VI intermediate filament protein, Nestin is part of the cytoskeleton. Due to its intracellular expression, it remains a challenge to isolate MSC for culture or even clinical therapy according to their Nestin expression.

Another interesting question would be to gain a more detailed understanding on the links between MSC and perivascular cells, mainly pericytes [241,242]. Pericytes are contractile cells that surround the endothelial cells of capillaries and venules throughout the entire body [243]. It is well known that cultured pericytes can give rise to MSC [244], but most probably not all pericytes are MSC [241,245]. Our technique would allow staining for all markers of both cell types in order to clarify this question.

5.7. Conclusions and relevance of the findings for MSC-based cell therapies

MSC have already been used without major safety concerns in many clinical trials, showing their efficacy for regenerative therapies in a range of acute or chronic ischemic tissue injuries to immune-mediated pathogenic processes [7]. Nevertheless, it has to be taken into account that there are reports showing clear evidence for alloimmunity to MSC in preclinical studies *in vitro* and *in vivo* [39,40,41]. For instance, Nauta et al. reported that allogeneic mouse MSC induced a memory T cell response when injected into naïve immunocompetent mice and stimulated donor graft rejection in a nonmyeloablative setting [39]. For this reason, it is very important to understand in detail how MSC modulate immune responses for their purpose. Even more importantly, in order to identify patients at risk, we need to understand under which circumstances MSC might provoke an alloresponse. Priming by transfusion of blood components (e.g. thrombocytes, leukocytes), organ or cell transplantation, pregnancy or simply by

unspecific cross-reactivity can lead to presensitization to alloantigens [246,247]. The preexistence of allospecific memory T or B cells or antibodies before as well as their development after MSC administration should be included in much more detail in future clinical trials. In our phase I/IIa study, even one patient who already showed a preexisting very low cross-reactivity towards donor PLX-PAD and unrelated third party donor cells at the beginning of the study, did not develop a clinically significant Th1 priming specific to PLX-PAD cells [177].

This thesis gives a more detailed understanding of the mode of action used by MSC to modulate DC and their crosstalk with NK and T cells. So far, conventional wisdom was that MSC efficiently suppress differentiation as well as maturation of DC precursors, not taking into account that diminished maturation might just be a consequence of the disturbed differentiation process. Here, we clearly show that MSC have diverse effects on the maturation of different DC subsets, depending on the type of DC and their time of interaction. MSC strongly interfere with the differentiation of human monocytes into moDC, which were shown to resemble rather inflammatory DC than conventional mDC [63,67,72]. Yet, *in vivo* they would not only encounter DC precursors but also already differentiated immature DC. Regarding maturation of different immature DC subpopulations (*in vitro* generated moDC, blood-derived pDC and mDC), MSC show almost no effect on the acquisition of maturation markers. However, pDC are not the main APC when thinking about application of allogeneic MSC. Therefore, we focussed on the influence of MSC on the mDC‘s functionality and could show that MSC very efficiently induce a tolerogenic phenotype, characterized by reduced migratory capacity, an anti-inflammatory cytokine profile, preserved antigen presentation and a strongly reduced capacity to activate NK and T cells. Particularly, this work undoubtedly points out how modification of DC functionality by MSC affects the complex and important interplay between DC, NK and T cells, which is indispensable for the initiation of immune responses, especially for lineage decision of naïve T cells towards the Th1 compartment [94,126].

Our findings are not only interesting when thinking about application of allogeneic MSC, but also for clinical settings in which autologous MSC are used. The control of checkpoints for Th1 priming is of special interest when thinking about MSC as cell

therapy for Th1-driven autoimmune and inflammatory diseases, such as GvHD, MS or systemic lupus erythematosus (SLE) [186]. GvHD is the major complication after HSC transplantation and arises when recipient APC activate donor T cells, leading to strong Th1 priming. There are already numerous studies showing that MSC are indeed able to attenuate or even prevent GvHD. MS is a severe disease, in which the myelin sheath of nerve cells in the brain and spinal cord are damaged by T cells. A shift from Th1 towards Th2 cytokine profile has been shown to be beneficial and many groups therefore investigate MSC as treatment option for MS patients [36,248]. Our findings emphasize that research in this direction is a step in the right direction. However, a lot of basic research needs to be done in order to understand the detailed beneficial effects of MSC in each clinical setting. Our staining for MSC in human tissue samples might be helpful in this context.

6. OUTLOOK

This work gives a more complete understanding about how MSC control checkpoints for Th1 priming. However, the molecular mechanism remains unclear. Since IL-10 was most probably secreted by mDC, the molecular mechanism by which MSC interact with DC remains an open question, which we would like to answer in the future. In the literature, PGE2 is considered as the most important MSC product interfering with DC functionality [137]. However, in our hands, PGE2 did not play a role (data not shown). One possible mechanism could be adenosine triphosphate (ATP) consumption by MSC, leading to energy deficiency. To get some hints, it would be interesting to investigate signalling pathways that are important for DC maturation, especially TLR4 signalling which leads to activation of different transcription factors, e.g. nuclear factor 'kappa-light-chain-enhancer' of activated B-cells (NFκB) or IRF3. In general, transcription factors of the IRF family are interesting to investigate in MSC-licensed DC. It has been shown that IRF5 promotes inflammatory M1 macrophage polarization and Th1 responses in mice by inducing transcription of IL-12p40 and IL-12p35 as well as by suppressing IL-10 expression, a cytokine profile reminding our BM-MSC-licensed mDC [249].

Bearing in mind that an “off-the-shelf” allogeneic cell product would be the most straightforward therapy option, our experimental settings could provide a test system to analyse and standardize the immunomodulatory capacity of different MSC cell products. This would give an opportunity to correlate the *in vitro* findings to data from preclinical and clinical trials.

A major future goal is to translate our knowledge about the interference with the interplay between DC, NK and T cells into patient samples in order to prove our model by *in vivo* data. Especially biopsies from the site of MSC injection could provide more details about this important interplay. They could be analysed by our recently developed technique for staining of MSC *in vivo*.

7. REFERENCES

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8. APPENDIX

List of publications

Consentius, C.; Reinke, P. and Volk, H. D. (2015): Immunogenicity of allogeneic mesenchymal stromal cells: what has been seen in vitro and in vivo?, Regen Med (vol. 10), No. 3, pp. 305-15.

Consentius, C.; Akyuz, L.; Schmidt-Lucke, J. A.; Tschope, C.; Pinzur, L.; Ofir, R.; Reinke, P.; Volk, H. D. and Juelke, K. (2015): Mesenchymal stromal cells prevent allostimulation in vivo and control checkpoints of Th1 priming: Migration of human DC to lymph nodes and NK cell activation, Stem Cells.

Selbstständigkeitserklärung

Hiermit erkläre ich, dass ich die vorliegende Arbeit „Inhibition of the crosstalk between dendritic, natural killer and T cells by mesenchymal stromal/stem cells “ selbständig verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel verwendet habe. Ich versichere, dass die Arbeit in dieser oder anderer Form noch keiner anderen Prüfungsbehörde vorgelegt wurde. Der Inhalt der Promotionsordnung der Mathematisch-Naturwissenschaftlichen Fakultät I der Humboldt Universität zu Berlin vom 06.07.2009 ist mir bekannt.

Berlin, den ....

(Christine Consentius)

Danksagung

Ich danke Herrn Prof. Dr. Hans-Dieter Volk für die Möglichkeit, meine Dissertation in seiner Arbeitsgruppe anzufertigen. Ich weiß die hilfreichen Anregungen, guten Ideen, Ratschläge und Menschlichkeit während dieser Zeit sehr zu schätzen.

Mein besonderer Dank gilt auch Frau Dr. Kerstin Jülke für die intensive Betreuung meiner Promotion. Deine außerordentliche fachliche und kritische Unterstützung haben mich wissenschaftlich geprägt und stark weiterentwickelt.

Herrn Prof. Dr. Michael Sittinger möchte ich herzlich für die Betreuung meiner Arbeit im Rahmen der Berlin-Brandenburg Schule für Regenerative Therapien (BSRT) danken.

Ein großer Dank geht an die gesamte AG Volk für die tolle Arbeitsatmosphäre und die konstruktiven Gespräche. Es hat immer viel Spaß gemacht mit euch zusammen zu arbeiten und ihr habt die Zeit meiner Promotion stark bereichert. Ganz besonders möchte ich Amelia, Jacky, Hong und Anke für viele unvergessliche Momente danken.

Als assoziiertes Mitglied der BSRT, möchte ich der Koordinatorin Dr. Sabine Bartosch für die interessanten Kurse und Weiterbildungsmöglichkeiten danken. Besonders möchte ich mich für die finanzielle Unterstützung im Rahmen der BSRT bedanken.

Ebenso bedanke ich mich sehr bei den anderen Arbeitsgruppen und „Core Facility“ Einrichtungen des BCRT und des Instituts für Medizinische Immunologie für die gute Zusammenarbeit. Insbesondere möchte ich mich bei Dr. Désirée Kunkel für die Hilfe beim Zellen sortieren bedanken. Frau Gabriela Korus möchte ich für die große Hilfsbereitschaft beim Zellkraftwerk-Projekt danken.

Ein ganz besonderer Dank gilt meiner Familie: meine Eltern Gerhild und Volker, die mich auf dem langen Weg der Ausbildung immer unterstützt haben, und meine Schwester Anne. Insbesondere meiner Mutter bin ich zutiefst dankbar, dass du immer an mich glaubst und meine wissenschaftliche Entwicklung voller Stolz begleitet hast.

Nicht zuletzt möchte ich meinem Mann Philipp danken. Ohne dich wäre diese Arbeit so nicht möglich gewesen. Bedingungslos hast du mich unterstützt und mir mit Nola und Mio unendliches Glück geschenkt. Ohne euch wären schwere Zeiten noch schwerer und schöne Zeiten sehr viel weniger schön gewesen.